Insights into the Enhanced *in vivo* Fitness of *Neisseria gonorrhoeae* Driven by a Fluoroquinolone Resistance-Conferring Mutant DNA Gyrase

by

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DEDICATION

This dissertation is dedicated to the memory of my grandmother.

The author hereby certifies that the use of any copyrighted material in the thesis manuscript entitled:

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ABSTRACT

Title of Dissertation:

"Insights into the Enhanced *in vivo* Fitness of *Neisseria gonorrhoeae* Driven by a Fluoroquinolone Resistance-Conferring Mutant DNA Gyrase"

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With no vaccine available, control of gonorrhea is seriously threatened by the evolution of heightened antibiotic resistance. The fluoroquinolone class of antibiotics served as a first-line treatment option for gonorrhea for only a limited period of time due to the proliferation of resistance mutations. Resistance to fluoroquinolones in *Neisseria gonorrhoeae* is achieved in a two-step process in which mutations in the quinolone resistance-determining regions of the genes encoding GyrA and ParC confer intermediate and full resistance, respectively. A clinically relevant $gyrA_{91/95}$ mutation was previously demonstrated by our laboratory to confer increased *in vivo* fitness to *N. gonorrhoeae* strain FA19, leading us to hypothesize that the resistance-conferring $gyrA_{91/95}$ mutations are sufficient to alter the *in vivo* fitness of other *N. gonorrhoeae* strains. We found that the presence of this allele in two additional strains of *N. gonorrhoeae* (FA1090 and

MS11) not only enhanced *in vivo* fitness, but did so at a level that was 10-fold higher than that reported in strain FA19. We further speculated that the enhanced *in vivo* fitness phenotype arose through the altered enzymatic behavior of mutant DNA gyrase, leading to a transcriptional profile better suited for bacterial survival or growth within the host. To test this hypothesis, transcriptome signatures were captured and compared between mutant and parent bacteria using total RNA sequencing as a means of identifying potential mechanisms of $gyrA_{91/95}$-driven enhanced *in vivo* fitness. *In vitro* assays were also conducted to compare wild-type and *gyrA* mutant bacterial phenotypes that might explain the enhanced fitness phenotype. We showed that genome-wide expression patterns differed across three sets of mutant and parent strains. Additionally, the mutant that showed the most robust enhancement in fitness relative to its parent strain was also more resistant to human and bacterial antimicrobial peptides, including the cathelicidins and H_2O_2 *in vitro*, and the fitness advantage of this mutant was not observed in cathelicidin-deficient mice. Collectively, these results suggest that a fluoroquinolone resistance-conferring mutations in GyrA condition the *N. gonorrhoeae* genome for increased survival or growth *in vivo*, and in the case of one strain background, these mutations increase resistance to host assault, allowing enhanced survivability over susceptible wild type bacteria.

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CHAPTER 1: Introduction

Neisseria gonorrhoeae

The majority of the 19 species within the genus *Neisseria* are human commensal bacteria. *Neisseria gonorrhoeae*, along with *Neisseria meningitidis* are the two species capable of causing disease in humans. First characterized by Albert Neisser in 1879, *N. gonorrhoeae* is a Gram-negative, non-motile, non-encapsulated diplococcus that has been exclusively adapted to the human host over thousands of years (81). Although other sites of infection exist, *N. gonorrhoeae* infections most commonly occur in the urogenital tract. With no external environmental or animal reservoir, *N. gonorrhoeae* has adapted to hostile host responses in humans through the evolution of mechanisms that counter these defenses. Antigenic modulation of the bacterial cell surface is a hallmark feature of *N. gonorrhoeae*, and this is one mechanism that helps the pathogen evade host defense mechanisms. Additional mechanisms include the capacity to evade innate effectors through surface structure modulation and the ability to utilize human iron-binding glycoproteins for efficient iron acquisition at iron-limited mucosal surfaces.

Epidemiology

N. gonorrhoeae infections are a global concern. The most recent data compilation from the World Health Organization on sexually transmitted infections reveals that over 106 million new cases of *N. gonorrhoeae* arose in 2008, which was estimated to be a

21% increase from three years prior (2). More recent estimates from the Center for Disease Control state that *N. gonorrhoeae* infections are the second most commonly reported infections in the United States. In 2012 alone, a total of 334,826 cases were reported in the U.S., representing a 4.1% increase from the year prior (1). The southern region of the United States carried the greatest burden of infection, followed by the Midwest, Northeast, and the Western parts of the country. Rates of infection are highest among adolescents and young adults, and tend to be slightly higher in women than men since 2001; however, the rate of increase in men was greater than 100-fold compared to that of women from 2011 to 2012 (1).

Pathogenesis

The urogenital epithelium are the primary site of *N. gonorrhoeae* infection. The uterine cervix and the urethra are the sites of initial infection in women and men, respectively. However, other common sites of infection include the pharynx and rectal mucosa, and in newborns, the conjunctiva. It is estimated that up to 4,000 newborn babies become blind annually worldwide due to eye infections resulting from untreated maternal gonococcal and chlamydial infections (2).

Gonococcal urogenital tract infections manifest differently in men and women. In men, a minor percentage of individuals may develop asymptomatic disease progression, but acute urethritis is the most common outcome. Influx of polymorphonuclear neutrophils (PMNs) and urethral epithelial cell shedding contribute to the mucopurulent discharge that is characteristic of symptomatic gonococcal disease and is the defining

feature of infection in men (26; 81). Symptomatic disease in men can lead to complications such as epididymitis, prostatitis, and infertility. In contrast, both symptomatic and asymptomatic cervical infections can lead to complications, and the overwhelming burden of asymptomatic infection is carried by women (26; 28; 32).

Gonococcal infection of women of child-bearing age most commonly presents as cervicitis. Unlike the singular hallmark feature gonococcal urethritis in men, there is significant variation in the clinical presentation of cervicitis (14; 26; 28). Features of infection may include vaginal bleeding, dysuria, vaginal discharge, erythema and edema, and menorrhagia. As high as 80% of women with cervical infections are asymptomatic (26). The common asymptomatic nature of gonococcal infection in women increases the likelihood of undiagnosed disease, which can facilitate progression to ascending infection in the upper genital tract. A major consequence of ascending gonococcal infection includes pelvic inflammatory disease (PID), which is often followed by long-term pelvic pain, ectopic pregnancy, and infertility. It is estimated that up to 45% of females with gonorrhea will develop PID. Furthermore, up to 90% of women with disseminated gonococcal infection present no indication of associated genital tract infection (26; 28; 32).

Virulence factors and evasion of host defenses

The genital mucosa contains a plethora of innate immune effectors that *N. gonorrhoeae* must successfully evade in order to establish infection in the human host.

Antimicrobial peptides, reactive oxygen species, and complement present challenges for effective gonococcal colonization.

Several virulence factors have been identified and studied in *N. gonorrhoeae* as mechanisms by which the gonococcus evades immune defenses. Lipooligosaccharide (LOS), pilus, and opacity-associated (Opa) outer membrane proteins are key contributors to the pathogen's successful adaptation to the human host (26; 30). Host immune evasion is enhanced through alterations in the expression of these surface-associated virulence factors, and the alterations are achieved through phase or antigenic variation (22; 105).

The biosynthesis of the gonococcal LOS is achieved through the activity of multiple LOS glycosyl transferase (*lgt*) genes resulting in a branched oligosaccharide molecule, which is anchored to the membrane by lipid A (74; 105). Three carbohydrate chains (α, β, and γ) display considerable variation, even within the same strain of *N. gonorrhoeae*, due to the high frequency of *lgt* gene phase variation (22; 88). Gonococcal LOS is involved in a range of activities that contribute to disease, including bacterial attachment to epithelial cells, the induction of cytokines and chemokines leading to PMN recruitment, and human fallopian tube damage (26). Modifications to LOS structure also promote gonococcal evasion of host defenses. For example, sialylation of gonococcal LOS is achieved through addition of host-derived neuraminic acid to the terminal galactose of the lacto-*N*-tetraose moiety of the LOS α-chain. Gonococcal LOS sialylation confers protection against LOS-specific antibodies and decreases susceptibility to human PMN killing (102).

Additionally, decoration of the 4' position of lipid A with phosphoethanolamine (PEA) confers increased resistance to complement and antimicrobial peptides. Like

gonococcal LOS sialylation, PEA decoration of lipid A confers protection against a range of host defenses. PEA can also be added to the heptose sugar within the β-chain of the primary oligosaccharide, but this modification does not affect serum resistance or resistance to antimicrobial peptides (50; 102). In contrast, PEA-decorated lipid A provides another mechanism of resistance to antimicrobial peptide killing (50; 63).

The gonococcal type IV pilus mediates attachment to non-ciliated secretory epithelial cells, provides motility for early bacterial distribution throughout the surface of the epithelium, and facilitates bacterial aggregation. Several genes encode various subunits of the type IV pili, and the PilE protein (encoded by *pilE*) is the major pilus subunit. Antigenic variation of PilE occurs as a result of recombination events between *pilE* and one of several copies of the silent *pilS* loci (54).

Another *N. gonorrhoeae* virulence factor that is involved in facilitating the interaction between the pathogen and host is the Opa protein family. These gonococcal surface proteins aid infection by elevating resistance to complement (19). Unlike other outer membrane surface components, the Opa protein family is thought to be responsible for gonococcal association with a broad range of host cell types. Like the gonococcal LOS, expression of Opa proteins is subject to phase variation (54). Up to 12 *opa* genes may be present within the genome of a particular strain of *N. gonorrhoeae* and as many as 4-5 proteins may be expressed simultaneously (38; 54). Sequence variability among Opa proteins is greatest within two central loops. These regions, referred to as hypervariable domain 1 (HV-1) and hypervariable domain 2 (HV-2), confer the tropism for certain cell types exhibited by different Opa proteins (38).

Efflux pump systems are present in most cells, and provide bacteria with a means of toxic elimination. Expulsion of a range of hydrophobic compounds including synthetic antibiotics and host-derived antimicrobial peptides eliminates or limits the lethality of these agents. Efflux pump systems fall into several families: the resistance-nodulation-cell division (RND) family, the ATP-binding cassette (ABC) family, the small multi-drug resistance family, the major facilitator (MF) family, and the multidrug and toxic compound extrusion (MATE) family (68). *N. gonorrhoeae* has four known efflux pumps including the FarA-FarB system (MF), NorM (MATE), the MacA/MacB system, and the multiple transferable resistance (MtrCDE) system, which is under the influence of an activator (MtrA) as well as a repressor (MtrR) (97). The FarA-FarB system recognizes and eliminates antimicrobial long-chain fatty acids. NorM provides the gonococcus with decreased susceptibility to several antibiotics that have quaternary ammonia structures (68). The MtrCDE system recognizes and exports certain antibiotics, hydrophobic agents, bile salts, steroidal hormones, and nonionic detergents (68). Susceptibility to host phagocytic or epithelial-induced cationic antimicrobial peptides/proteins (CAMP) is decreased in *N. gonorrhoeae* through the activity of the MtrCDE efflux pump.

The migration of PMNs to the site of infection, engulfment of bacteria, and elimination of microbes during an inflammatory response involves mechanisms that employ oxygen-dependent killing. Activation of PMNs leads to an oxidative burst catalyzed by the activity of NADPH oxidase within the phagosome membrane; however, *N. gonorrhoeae* is able to survive and replicate within host PMNs (82).

N. gonorrhoeae has several antioxidant systems. For example, production of gonococcal catalase has been shown to be critical for H_2O_2 tolerance *in vitro* and the MntABC-type transporter uses the manganous ion (Mn^{2+}) as a means of quenching reactive oxygen species (ROS) (71; 72). The gonococcal cytochrome c peroxidase and methionine sulfoxide reductase enzymes both provide protection against H_2O_2 killing (71; 72). However, while clearly important in the protection against oxidative stress *in vitro*, none of the mechanisms described above lead to evasion of PMN-produced ROS (103) (39; 79).

Experimental models of infection

Since natural infection with *N. gonorrhoeae* is exclusive to the human host, investigating the dynamics of pathogenesis from both the host and bacterial perspective presents challenges. Studies with male volunteers have provided a wealth of insight into the early stages of urethral infection. These studies have also facilitated the understanding of gonococcal virulence factors and the susceptibility to reinfection as well as provided information regarding vaccine candidates. However, these studies are limited to the urethral epithelium in males, and events that define the course of infection in females cannot be implied. Primary human cell cultures have been utilized to investigate gonococcal involvement in the upper and lower genital tracts, yet, these cell culture systems are restricted to cell type and fail to encapsulate the range of pathogenesis that occurs throughout the female genital tract.

Early animal models of gonococcal infection included the extragenital inoculation of rabbits and mice in the 1930s and 1940s; however, the use of animals to study gonococcal disease ceased throughout the middle part of the 20th century (8; 9; 21). Investigations of gonococcal arthritis and disseminated infection were enhanced through the use of rabbits and small rodents throughout the 1970s and 1980s (11), and while these models provided valuable insight into the nature of extragenital gonococcal infections, there were no animal models of gonococcal genital tract infection until chimpanzees were utilized in pathogenesis and sexual transmission studies (10). Unfortunately, the use of non-human primates presents considerable fiscal constraints, which has led some investigators to pursue murine models of gonococcal infection.

Studies in the 1980s provided evidence that female mice could be infected with *N. gonorrhoeae* during proestrous, which is a 12-24 hour period of increasing estrogen during the murine estrous cycle (41). This window of susceptibility was extended through the use of 17β-estradiol injections to establish prolonged gonococcal infection in gnotobiotic BALB/c female mice (86). In 1999, our laboratory confirmed the findings of this effort and refined the technique with the use of antibiotics to eliminate the inhibitory influence of vaginal flora. In our current protocol, which uses a series of three subcutaneous injections of water-soluble estradiol, colonization is maintained in female BALB/c mice for an average of 10 days (41). Following vaginal inoculation with approximately 10^6 colony forming units (CFUs) of *N. gonorrhoeae*, an average of 10^3 to 10^5 CFUs are recovered from a single vaginal swab. It is not known exactly why estradiol is required for long-term colonization in mice; however, suppression of PMN

influx, alteration of immune receptors, and a diminished cytokine production may be responsible (41).

Diagnosis and Treatment

Nucleic Acid Amplification Tests (NAATs) are recommended for the detection of *N. gonorrhoeae* genital tract infections in men and women. Vaginal swabs from women and first catch urine from men are the specimens of choice for NAAT-based detection of *N. gonorrhoeae* (23). Although not cleared by the Food and Drug Administration for extragenital sites, NAAT detection is utilized in the clinical laboratory with established performance specifications under the Clinical Laboratory Improvement Amendments (CLIA) regulatory guidance. Traditional culture methods are employed when antimicrobial susceptibility testing is needed in the case of treatment failure. Culture-based detection is also needed in cases of possible sexual assault in children (23).

The management of gonorrhea infection rests exclusively on antibiotic treatment. Prior to the success of sulfa drug treatment for gonococcal infections in the late 1930's, urethritis in men and cervicitis in women were often treated with cumbersome injections of potassium permanganate and organic silver salt into the urethra and vagina (43). Antibiotic treatment first began with sulfonamide circa 1937, and this approach was followed by the penicillin era in the early 1940s, which led to a significant decline in gonococcal infections (43). However, in the forty years that have followed, *N. gonorrhoeae* has established itself as an organism with the capacity to develop resistance to all antibiotics that have thus far been recommended for treatment. Now, with

resistance emerging to the third-generation cephalosporins, a dual regimen of injectable ceftriaxone in combination with one of two oral antibiotics, either azithromycin or doxycycline, as the lone treatment option for gonorrhea (1).

Gonococcal Vaccine Development

The global prevalence of gonococcal infections, continued proliferation of multi-class antibiotic resistance, and the devastating sequelae associated with asymptomatic infections in women demand a need for gonococcal vaccine development. However, several factors have contributed to a relatively conservative approach to *N. gonorrhoeae* vaccine research. While a significant health concern, gonorrhea may not be viewed by many as a threat of the level of other diseases. Gonococcal infections lack the high profile lethality of meningococcal disease, which may limit the wide-spread public backing needed to help elevate research toward a gonococcal vaccine. Additional factors that have limited research progression in this field include the highly variable nature of *N. gonorrhoeae* surface components as discussed earlier, a lack of evidence in support of naturally-acquired immunity, and undefined correlates of protection (18; 24; 40).

To date, the few gonococcal vaccines that have advanced to clinical trials have been unsuccessful. The utility of a killed whole cell vaccine was investigated on a population in Northern Canada (33; 34). However, no protection to subsequent challenge was observed, and further exploration of whole cell gonococcal vaccines ceased. As mentioned previously, the gonococcal type IV pili mediate attachment, afford motility, and mediate bacterial aggregation during infection. Considerable attempts aimed at

developing a purified pilin vaccine showed early promise. Studies in human volunteers generated an immunogenic response, with blocking antibodies observed in both serum and genital secretions (53; 76; 89). Using a *N. gonorrhoeae* pilin vaccine, a large-scale field trial was carried out in the Republic of South Korea on 3123 male and 127 female U.S. military active duty volunteers (20). The study led to no evidence of protection, and the failure of this single-antigen vaccine trial has been attributed to the highly variable nature of the gonococcal pilin. No additional vaccine candidates have advanced to clinical trials since the failures of the whole cell killed and pilin vaccines.

The absence of any promising vaccine candidates currently in product pipeline and the accelerating risk of untreatable gonorrhea through the spread of ceftriaxone resistance provides a formidable challenge to public health.

Antibiotic Resistance

Just as the sulfonamides were fading as a successful treatment option, the discovery of penicillin and its effectiveness as a therapy for gonococcal infections ushered in a new treatment era (95). The accidental discovery of penicillin in the late 1920's by Alexander Fleming was slow to catch on as a treatment for gonococcal infections, but by 1943 the use of penicillin was an efficient and effective means for managing gonorrhea infection. However, not long after surpassing the sulfonamides as the means of primary therapy for gonorrhea, penicillin resistant determinants started to emerge. The development of later classes of antibiotics, such as the macrolides, aminoglycosides, and the tetracyclines, provided a means of circumventing intolerance to

penicillin in some infected individuals; however, by the time penicillin resistance became wide-spread and was no longer recommended for the treatment of gonorrhea, *N. gonorrhoeae* had also acquired either plasmid or chromosomally-mediated resistance mutations to several of these antibiotics, including streptomycin erythromycin, tetracycline, and spectinomycin (52; 78; 87; 96).

Penicillins disrupt the dynamic process of bacterial cell wall metabolism; however, *N. gonorrhoeae* has acquired a diverse set of chromosomally mediated and plasmid-encoded determinants that confer resistance to penicillins. Mutations in the *penA* and *ponA* genes, which encode the primary and secondary targets of penicillin, penicillin binding proteins 2 and 1 (PBP2 and PBP1) respectively, lead to decreased penicillin-directed acylation (95; 96). Mutations that lead to over-expression of the MtrCDE efflux pump lead to decreased drug sequestration. Reduction in penicillin influx is also associated with *porB1b* single nucleotide polymorphisms (SNPs) and *pilQ* mutations that disrupt the formation of the type IV pilus. The TEM-1 and TEM-135 penicillinase-encoding plasmids directly inactivate penicillin through an attack on the β-lactam ring. Finally, "Factor X" is an unknown resistant determinant associated with up to 6-fold increases in penicillin Minimum Inhibitory Concentrations (MICs) (61; 95; 96).

As mentioned, the tetracycline class of antibiotics provided an effective means of treating gonorrhea for individuals with penicillin sensitivities. Tetracycline targets the 30S ribosomal subunit; however, chromosomal mutations in the *rpsJ* gene of *N. gonorrhoeae* lead to diminished target affinity. Acquisition of *tetM*-encoding plasmids further increases MICs to tetracycline by producing an elongation factor-like protein that prevents drug association (95; 96). Like penicillin resistance, increased MtrCDE efflux

pump expression and *pilQ* and *penB* resistance determinants that decrease drug influx also raise tetracycline MICs.

Like tetracycline, spectinomycin blocks bacterial protein translation through an inhibitory association with the 30S ribosomal subunit. Specifically, inhibition of bacterial elongation factor G (EF-G) is achieved by direct association of spectinomycin with the 16S rRNA (94). Mutations in the gonococcal *rpsE* gene that alter the amino acid sequence of the S5 ribosomal protein decrease drug binding (52; 87; 96) .

Resistance to macrolides, which inhibit bacterial protein synthesis through 50S subunit targeting, is achieved in the gonococcus through decreased drug association with the ribosomal complex. This decreased drug-target association occurs via mutations in the 23S rRNA that alter the amino acid composition, or through the acquisition of macrolide-lincosamide-streptogramin B resistance genes (*erm*), which encode rRNA methylases (57). The methylation of 23S rRNA masks the macrolide binding site. Mutations that lead to de-repression of the MtrCDE efflux pump also raise macrolide MICs (25; 96).

The development of the broad-spectrum fluoroquinolone class of antibiotics emerged as an attractive therapeutic alternative in the wake of resistant strains of bacteria in the early 1980s (27) (95). This bactericidal class of antibiotic targets Type II topoisomerases in the cell and inhibits the enzyme in a mechanism thought to involve a DNA-bound complex (5; 64; 104). The prevailing model of inhibition involves binding of the fluoroquinolone molecules to the DNA enzyme complex and accumulation of a cleaved DNA-enzyme lethal intermediate (104). By the mid-1980s, ciprofloxacin became a popular antibiotic therapy for *N. gonorrhoeae* infections after the CDC reported

rising numbers of plasmid-mediated resistance to tetracycline and penicillinase-producing gonococcal isolates (55). Unfortunately, although highly effective in the treatment of gonorrhea, increased MICs to ciprofloxacin were being reported in Southeast Asia as early as 1988. The first clinically resistant isolates of *N. gonorrhoeae* were reported in Australia, and by the late 1990s, the percentage of clinically resistant isolates in Hawaii rose to 9.5% (22 of 231) (91; 95). By 2000, over 80% of the gonococcal isolates from Western Pacific Region were clinically resistant to ciprofloxacin. Clinically resistant isolates continued to move west, and by 2007, 14.5% (891 of 6009) of gonococcal isolates in the United States were clinically resistant (56; 91), and in 2007, the CDC discontinued the recommendation of fluoroquinolones as a primary treatment option for gonorrhea. In summary, resistance to the fluoroquinolones occurred after a relatively short duration of recommended use, and the loss of fluoroquinolone use exemplifies the rapid emergence of antibiotic resistance in the gonococcus (45).

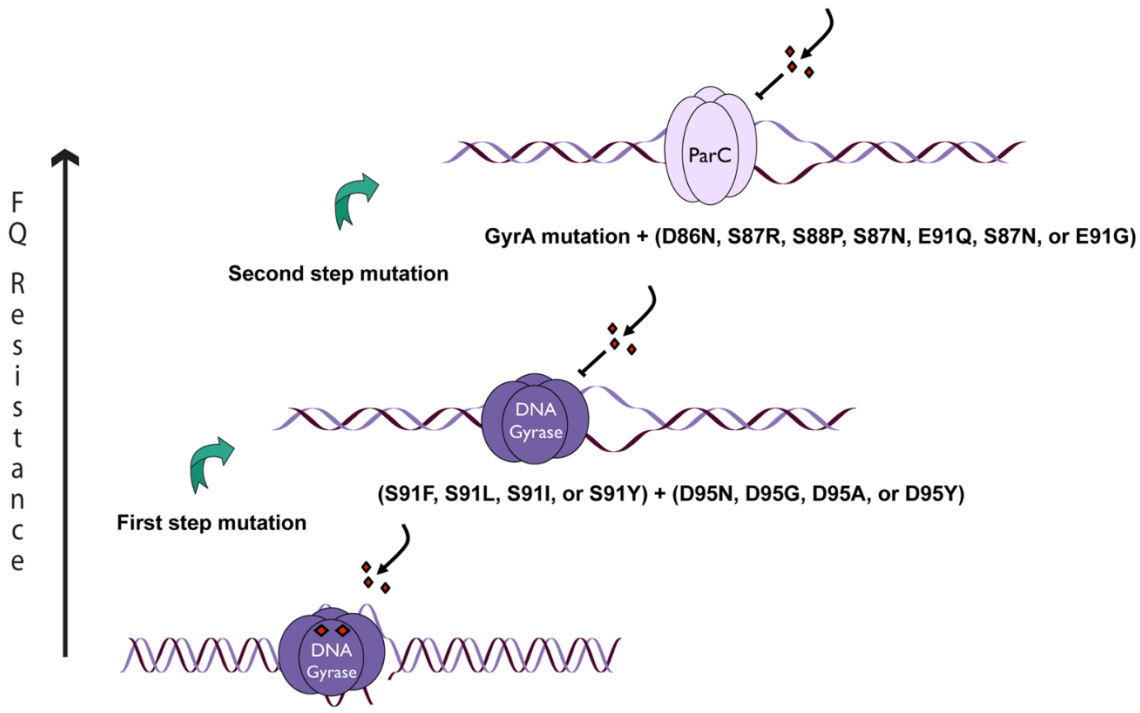
Type II Topoisomerase and the Quinolone Resistance-Determining Region

As mentioned above, the targets of fluoroquinolones are the heterotetramer Type II topoisomerase enzymes DNA gyrase and topoisomerase IV. The genes encoding the individual subunits of these heterotetramers are *gyrA* and *gyrB* (DNA gyrase) and *parC and parE* (topoisomerase IV) (90; 101). The state of genomic topology plays an important role during the key molecular events of transcription and DNA replication, where supercoiling of the DNA is altered to accommodate these biological processes (77). The type II topoisomerases are responsible for managing the genomic topology in

the bacterial cell, and this is achieved by an ATP-dependent, double-stranded cleavage of the DNA duplex. While both DNA gyrase and topoisomerase IV share the same mechanistic approach for influencing the degree of DNA supercoiling, DNA gyrase functions primarily to increase negative supercoiling, while topoisomerase IV is mainly involved in the separation of sister chromatids during DNA replication (15). As mentioned previously, it took less than 20 years before the fluoroquinolone class of antibiotics was no longer a first-line treatment option for *N. gonorrhoeae*. Emergence of fluoroquinolone resistance in *N. gonorrhoeae* was due to acquisition of chromosomal mutations in the quinolone resistance-determining regions (QRDRs) within the *gyrA* and *parC* genes (64; 73; 83; 85; 92; 93; 106). Acquisition of these mutations occurs in a stepwise manner (**Fig. 1**), with a double point mutation in *gyrA* conferring an intermediate degree of resistance to ciprofloxacin (Cip^I) (95). An additional single point mutation in *parC* leads to high-level resistance to ciprofloxacin (Cip^R).

Figure 1. Acquisition of intermediate level resistance (CipI) and high level resistance (CipR) to fluoroquinolones in N. gonorrhoeae occurs in a step-wise manner.

This illustration represents the steps leading to increased resistance to fluoroquinolones in *N. gonorrhoeae* with an emphasis on its target (DNA-bound topoisomerase). The susceptible (wild-type) state is represented at the bottom of the figure in which wild-type DNA gyrase is bound and inhibited by fluoroquinolones. A key feature highlighted at the bottom of the figure include the direct association of fluoroquinolone molecules (♦) with the nonfunctional DNA gyrase and cleaved DNA duplex intermediate. The images representing DNA gyrase in Cip^I and Cip^R strains highlight the lack of association between antibiotic and enzyme. Intermediate-level resistance is most commonly achieved through the acquisition of a double point mutation in *gyrA* resulting in one of the more frequent amino acid substitutions highlighted. An additional single point mutation in *parC* establishes a high degree of resistance, and commonly encountered amino acid substitutions associated with this mutant are displayed (59).


FQ Resistance
ParC
GyrA mutation + (D86N, S87R, S88P, S87N, E91Q, S87N, or E91G)
Second step mutation
DNA Gyrase
(S91F, S91L, S91I, or S91Y) + (D95N, D95G, D95A, or D95Y)
First step mutation
DNA Gyrase

Bacterial Fitness

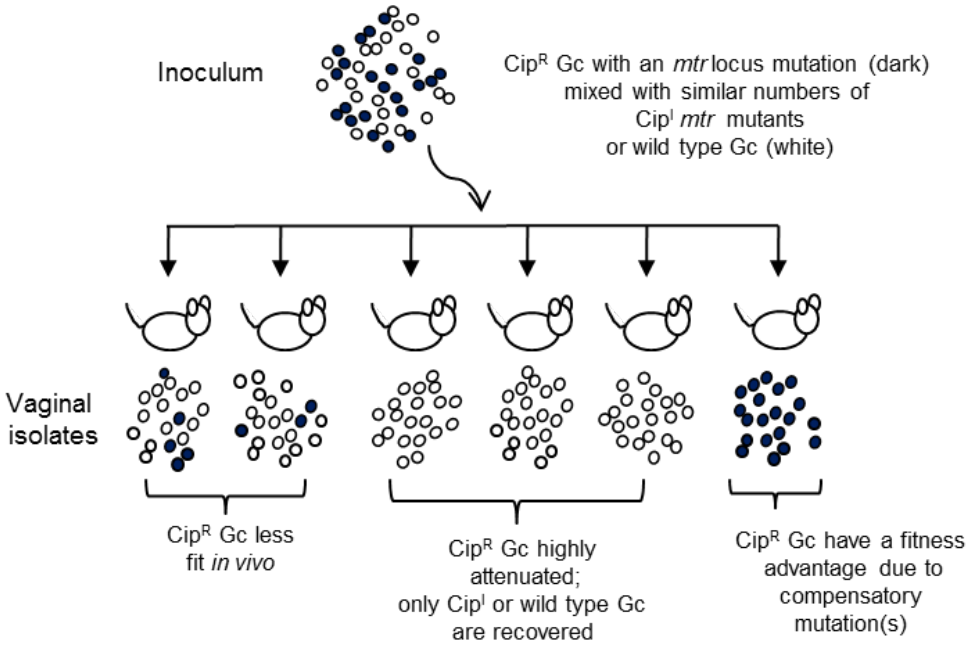
Bacterial mutations that decrease the cell's sensitivity to antibiotics have generally been shown to negatively impact bacterial fitness (3; 6; 7; 13). Fanning and colleagues characterized fitness costs in *Salmonella enterica* resulting from high-level resistance to ciprofloxacin and identified reduced growth rates, motility, and invasiveness as phenotypes in the resistant mutant that might provide a barrier to survival when the antibiotic selective pressure is removed (58). However, studies that link intermediate level fluoroquinolone resistance-conferring mutations with an *in vivo* fitness advantage have also been reported. In one study, fluoroquinolone-resistant *Campylobacter jejuni* mutants carrying a single point mutation in the *gyrA* allele outcompeted fluoroquinolone-sensitive strains of the same genetic background in an *in vivo* chicken model (51). Further studies demonstrated that this point mutation also altered changes in supercoiling (51) (37), although how this observation may cause increased *in vivo* fitness has not been explained. Studies in *E. coli* demonstrated that high level fluoroquinolone resistance-conferring mutations in *gyrA* and *parC* produced alterations in both growth rate and global supercoiling (12). Furthermore, and similar to the *C. jejuni* study, ciprofloxacin resistance-conferring mutations in the *E. coli gyrA* gene were recently shown to alter the state of genomic supercoiling and the *E. coli* transcriptome, with several of these changes affecting the expression levels of genes involved in the stress response (100).

In *N. gonorrhoeae*, we reported that Cip^I mutants display an enhanced *in vivo* phenotype in a female mouse model of experimental gonococcal genital tract infection. Using the common laboratory strain of *N. gonorrhoeae*, FA19, we showed that Cip^I

mutants carrying commonly isolated *gyrA*$_{91/95}$ mutations have a fitness advantage when competed against wild type Cip^S bacteria. However, this *in vivo* fitness advantage is lost with the acquisition of a common second-site mutation in the *parC* gene that is required for high-level resistance to ciprofloxacin (47). Our laboratory also demonstrated the selection of a compensatory mutation during an *in vivo* competitive infection between Cip^I and Cip^R strains that also carry an *mtr* antibiotic resistance mutation. This result provides evidence that in-host microbial evolution can provide a mechanism for circumventing the fitness costs while maintaining high-level resistance to antibiotics in the absence of selective pressure. The *in vivo*-selected Cip^R mutant with an enhanced growth rate relative to parent strain was sequenced and shown to have alterations in *gyrA* (S91L) and a repaired promoter for a global regulator (*mtrR*). Interestingly, the *gyrA* (S91L) mutation has been reported in clinical isolates of Cip^R *N. gonorrhoeae* (47) (97; 98). A schematic illustrating the competitive infection procedure and the results described above is shown in **Fig. 2**.

As we continue to move toward an era of untreatable bacterial infections, efforts to establish a fitness value for different resistance-conferring mutations may help predict spread of certain types of resistances. Furthermore, continued characterization of the mechanisms that lead to either a fitness advantage or disadvantage over isogenic sensitive strains may help influence efforts in targeted drug discovery as well as further our understanding of gonococcal adaptation to the host.

Figure 2. Fitness studies with Cip^R *N. gonorrhoeae* using the mouse model of experimental infection. Results showed that the CipR mutant carrying an additional antibiotic resistance mutation in the mtr locus exhibited a fitness disadvantage relative to CipI or CipS bacteria. Restored or enhanced fitness can be achieved through compensatory mutations (59).


Inoculum
Cip^R Gc with an *mtr* locus mutation (dark) mixed with similar numbers of Cip^I *mtr* mutants or wild type Gc (white)
Vaginal isolates
Cip^R Gc less fit *in vivo*
Cip^R Gc highly attenuated; only Cip^I or wild type Gc are recovered
Cip^R Gc have a fitness advantage due to compensatory mutation(s)

Hypothesis and Goals

Building on previously reported evidence from our laboratory that *N. gonorrhoeae* carrying a mutant $gyrA_{91/95}$ allele possesses a fitness advantage over wild type sensitive bacteria, the goal of this work was to expand the investigation of the observed enhanced *in vivo* fitness across multiple strains and to identify the basis of this enhanced fitness by examining transcriptional profiles and conducting *in vitro* and *in vivo* assays to measure bacterial resistance to host defenses.

Due to the rapid emergence of fluoroquinolone reistance in *N. gonorrhoeae*, we hypothesized that fluoroquinolone resistance-conferring $gyrA_{91/95}$ allele of *N. gonorrhoeae* imparts a strain-independent *in vivo* fitness advantage. We also hypothesized that this mutation alters the global expression of bacterial genes that results in increased gonococcal survival *in vivo*.

CHAPTER 2: Materials and Methods

Bacterial strains and mutant construction

Neisseria gonorrhoeae strain FA19Sm^R and its *gyrA*$_{91/95}$ mutant AK1 were described previously (47). *N. gonorrhoeae* strains FA1090 and MS11 were used to create *gyrA*$_{91/95}$ mutants JD1and JD4, respectively. JD1 and JD4 *gyrA*$_{91/95}$ mutants were constructed by allelic exchange with a donor PCR amplicon containing Ser91Phe (TCC to TTC) and Asp95Asn (GAC to AAC) mutations (**Table 1**). The method employed followed that previously reported for the construction of AK1 (35; 47). Briefly, a 1.3-kb region of *gyrA* was amplified from AK1 using gyrA1-for and gyrA1-rev primers (**Table 2**). The resultant amplicon was gel-extracted, and sequenced to confirm the mutated sequence. FA1090 and MS11 bacteria were suspended in PBS and adjusted to an absorbance of 0.05 at 600 nanometers (A_{600}). Ten microliters of the bacterial suspension were spotted onto solid non-selective gonococci (GC) agar base media followed by direct overlay of approximately 1 µg of the donor DNA. Following incubation at 37º C for 24 hours, individual colonies were isolated and streaked onto solid agar containing 0.125 µg/ml of ciprofloxacin to select for *gyrA*$_{91/95}$ mutants. The presence of the desired mutation in colonies that grew on the ciprofloxacin plates was confirmed by sequence analysis (**Figures 3 and 4**).

Table 1. List of parent strains and mutants used throughout these studies.

The relevant genotype, fluoroquinolone resistance phenotype, and minimum inhibitory concentrations (MICs) are shown for each parent and mutant strain.

Strain	Genotype	Phenotype	MIC (Cip) (µg/ml)
FA19Sm^R	Spontaneous Sm^R mutant of wild-type FA19	Cip^S	< 0.06
AK1	*gyrA*$_{91/95}$ mutant of FA19Sm^R	Cip^I	0.25
AK2	*gyrA*$_{91/95}$; *par*C$_{86}$ mutant of FA19Sm^R	Cip^R	6
FA1090	*mtrA*$^-$	Cip^S; decreased *mtrC-D-E* activation	< 0.06
JD1	*gyrA*$_{91/95}$ mutant of FA1090	Cip^I; decreased *mtrC-D-E* activation	0.38
JD1.2	*gyrA*$_{91/95}$; *par*C$_{86}$ mutant of FA1090	Cip^R; decreased *mtrC-D-E* activation	6
MS11	Wild-type strain, *mtr*$_{120}$, *mtrR*$_{A39T}$	Cip^S; naturally de-repressed *mtrC-D-E*	< 0.06
JD4	*gyrA*$_{91/95}$ mutant of MS11	Cip^I; naturally de-repressed *mtrC-D-E*	1.5
JD4.2	*gyrA*$_{91/95}$; *parC*$_{86}$ mutant of MS11	Cip^R; naturally de-repressed *mtrC-D-E*	8

Table 2. Oligonucleotide primers used for strain construction and sequencing (5’ to 3’)

gyrA1-for	GACTTCCTCATGCAGCAAATG
gyrA1-rev	CAACCATATTGATGCCGAAACTG
gyrA2-for	ACGAAACATTGAAACCATGAC
parC1-for	CATAGCGACGGTCTTTGTGTG
parC1-rev	GTTGATGAAGGTATCGGTATCGATG
parC2-for	ACGCTTCCCATACCGATTC
5’mtrR	GGTTAATTAACGCCTTAGAAGCATAAAAAGC
3’mtrR	GGGTTTAAACTTATTTCCGGCGCAGGCAG

Figure 3. *gyrA* nucleotide sequence from mutant JD1.

The highlighted codons show the TCC → TTC point mutation that produces the Ser → Phe amino acid transition at position 91 (S91F) and the GAC → AAC point mutation that leads to the Asp → Asn amino acid change at position 95 (D95N).

ATGACCGACGCAACCATCCGCCACGACCACAAATTCGCCCTCGAAACCCTGCCCGTCAGCCTTGAAGACG
AAATGCGCAAAAGCTATCTCGACTACGCCATGAGCGTCATTGTCGGGCGCGCGCTGCCGGACGTTCGCGA
CGGCCTAAAGCCGGTGCACCGGCGCGTACTGTACGCGATGCACGAGCTGAAAAATAACTGGAATGCCGCC
TACAAAAAATCGGCGCGCATCGTCGGCGACGTCATCGGTAAATACCACCCCCACGGCGATTTCGCAGTTT
ACAACACCATCGTCCGTATGGCGCAAAATTTCGCTATGCGTTATGTGCTGATAGACGGACAGGGCAACTT
CGGATCGGTGGACGGGCTTGCCGCCGCAGCCATGCGCTATACCGAAATCCGCATGGCGAAAATCTCACAT
GAAATGCTGGCAGACATTGAGGAAGAAACCGTTAATTTCGGCCCGAACTACGACGGTAGCGAACACGAGC
CGCTTGTACTGCCGACCCGTTTCCCCACACTGCTCGTCAACGGCTCGTCCGGTATCGCCGTCGGTATGGC
GACCAACATCCCGCCGCACAACCTCACCGACACCATCAACGCCTGTCTGCGTCTTTTGGACGAACCCAAA
ACCGAAATCGACGAACTGATCGACATTATCCAAGCCCCCGACTTCCCGACCGGGGCAACCATCTACGGCT
TGGGCGGCGTGCGCGAAGGCTATAAAACAGGCCGCGGCCGCGTCGTTATACGCGGTAAGACCCATATCGA
ACCCATAGGCAAAAACGGCGAACGCGAAGCCATCGTTATCGACGAAATCCCCTATCAGGTCAACAAAGCC
AAGTTGGTCGAGAAAATCGGCGATTTGGTTCGGGAAAAAACGCTGGAAGGCATTTCCGAGCTCCGCGACG
AATCCGACAAATCCGGGATGCGCGTCGTTATCGAGCTGAAACGCAACGAAAATGCCGAAGTCGTCTTAAA
CCAACTCTACAAACTGACTCCGCTGCAAGACAGTTTCGGCATCAATATGGTTGTTTTGGTCGACGGACAA
CCGCGCCTGTTAAACCTGAAACAGATTCTCTCCGAATTCCTGCGCCACCGCCGCGAAGTCGTTACCCGAC
GTACGCTTTTCCGGCTGAAGAAGGCACGCCATGAAGGGCATATCGCCGAAGGCAAAGCCGTCGCACTGTC
CAATATCGATGAAATCATCAAGCTCATCAAAGAATCGCCCAACGCGGCCGAGGCCAAAGAAAAACTGCTT
GCGCGCCCTTGGCGCAGCAGCCTCGTTGAAGAAATGCTGACGCGTTCCGGTCTGGATTTGGAAATGATGC
GTCCGGAAGGATTGGCTGCAAACATTGGTCTGAAAAAACAAGGTTATTACCTGAGCGAGATTCAGGCAGA
TGCTATTTTACGCATGAGCCTGCGAAACCTGACCGGCCTCGATCAGAAAGAAATTATCGAAAGCTACAAA
AACCTGATGGGTAAAATCATCGACTTTGTGGATATCCTCTCCAAACCCGAACGCATTACCCAAATCATCC
GTGACGAACTGGAAGAAATCAAAACCAACTATGGCGACGAACGCCGCAGCGAAATCAACCCGTTCGGCGG
CGACATTGCCGATGAAGACCTGATTCCGCAACGCGAAATGGTCGTGACCCTGACCCACGGCGGCTATATA
AAAACCCAGCCGACCACCGACTATCAGGCTCAGCGTCGCGGCGGGCGCGGCAAACAGGCGGCTGCCACCA
AAGACGAAGACTTTATCGAAACCCTGTTTGTTGCCAACACGCATGACTATTTGATGTGTTTTACCAACCT
CGGCAAGTGCCACTGGATTAAGGTTTACAAACTGCCCGAAGGCGGACGCAACAGCCGCGGCCGTCCGATT
AACAACGTCATCCAGCTGGAAGAAGGCGAAAAAGTCAGCGCGATTCTGGCAGTACGCGAGTTTCCCGAAG
ACCAATACGTCTTCTTCGCCACCGCGCAGGGAATGGTGAAAAAAGTCCAACTTTCCGCCTTTAAAAACGT
CCGCGCCCAAGGCATTAAAGCCATCGCACTCAAAGAAGGCGACTACCTCGTCGGCGCTGCGCAAACAGGC
GGTGCGGACGACATTATGTTGTTCTCCAACTTGGGCAAAGCCATCCGCTTCAACGAATACTGGGAAAAAT
CCGGCAACGACGAAGCGGAAGATGCCGACATCGAAACCGAGATTTCAGACGACCTCGAAGACGAAACCGC
CGACAACGAAAACACCCTGCCAAGCGGCAAAAACGGCGTGCGTCCGTCCGGTCGCGGCAGCGGCGGTTTG
CGCGGTATGCGCCTGCCTGCCGACGGCAAAATCGTCAGCCTGATTACCTTCGCCCCTGAAACCGAAGAAA
GCGGTTTGCAAGTTTTAACCGCCACCGCCAACGGATACGGAAAACGCACCCCGATTGCCGATTACAGCCG
CAAAAACAAAGGCGGGCAAGGCAGTATTGCCATTAACACCGGCGAGCGCAACGGCGATTTGGTCGCCGCA
ACCTTGGTCGGCGAAACCGACGATTTGATGCTGATTACCAGCGGCGGCGTGCTTATCCGTACCAAAGTCG
AACAAATCCGCGAAACCGGCCGCGCCGCAGCAGGCGTGAAACTGATTAACTTGGACGAAGGCGAAACCTT
GGTATCGCTGGAACGTGTTGCCGAAGACGAATCCGAACTCTCCGGCGCTTCTGTAATTTCCAATGTAACC
GAACCGGAAGCCGAGAACTGA

Figure 4. *gyrA* nucleotide sequence from mutant JD4.

The highlighted codon shows the TCC → TTC point mutation that produces the Ser → Phe amino acid transition at position 91 (S91F) and the GAC → AAC point mutation that leads to the Asp → Asn amino acid change at position 95 (D95N).

ATGACCGACGCAACCATCCGCCACGACCACAAATTCGCCCTCGAAACCCTGCCCGTCAGCCTTGAAGACG
AAATGCGCAAAAGCTATCTCGACTACGCCATGAGCGTCATTGTCGGGCGCGCGCTGCCGGACGTTCGCGA
CGGCCTAAAGCCGGTGCACCGGCGCGTACTGTACGCGATGCACGAGCTGAAAAATAACTGGAATGCCGCC
TACAAAAAATCGGCGCGCATCGTCGGCGACGTCATCGGTAAATACCACCCCCACGGCGATTTCGCAGTTT
ACAACACCATCGTCCGTATGGCGCAAAATTTCGCTATGCGTTATGTGCTGATAGACGGACAGGGCAACTT
CGGATCGGTGGACGGGCTTGCCGCCGCAGCCATGCGCTATACCGAAATCCGCATGGCGAAAATCTCACAT
GAAATGCTGGCAGACATTGAGGAAGAAACCGTTAATTTCGGCCCGAACTACGACGGTAGCGAACACGAGC
CGCTTGTACTGCCGACCCGTTTCCCCACACTGCTCGTCAACGGCTCGTCCGGTATCGCCGTCGGTATGGC
GACCAACATCCCGCCGCACAACCTCACCGACACCATCAACGCCTGTCTGCGTCTTTTGGACGAACCCAAA
ACCGAAATCGACGAACTGATCGACATTATCCAAGCCCCCGACTTCCCGACCGGGGCAACCATCTACGGCT
TGGGCGGCGTGCGCGAAGGCTATAAAACAGGCCGCGGCCGCGTTGTTATGCGCGGTAAGACCCATATCGA
ACCCATAGGCAAAAACGGCGAACGCGAAGCCATCGTTATCGACGAAATCCCCTATCAGGTCAACAAAGCC
AAGTTGGTCGAGAAAATCGGCGATTTGGTTCGGGAAAAAACACTGGAAGGCATTTCCGAGCTCCGCGACG
AATCCGACAAATCCGGTATGCGCGTCGTTATCGAGCTGAAACGCAACGAAAATGCCGAAGTCGTCTTAAA
CCAACTCTACAAACTGACTCCGCTGCAAGACAGTTTCGGCATCAATATGGTGGTTTTGGTCGACGGACAA
CCGCGCCTGTTAAACCTGAAACAGATTCTCTCCGAATTCCTGCGCCACCGCCGCGAAGTCGTTACCCGAC
GTACGCTTTTCCGGCTGAAGAAGGCACGCCATGAAGGGCATATCGCCGAAGGCAAAGCCGTCGCACTGTC
CAATATCGATGAAATCATCAAGCTCATCAAAGAATCGCCCAACGCGGCCGAGGCCAAAGAAAAACTGCTT
GCGCGCCCTTGGCGCAGCAGCCTCGTTGAAGAAATGCTGACGCGTTCCGGTCTGGATTTGGAAATGATGC
GTCCGGAAGGATTGGCTGCAAACATTGGTCTGAAAAAACAAGGTTATTACCTGAGCGAGATTCAGGCAGA
TGCTATTTTACGCATGAGCCTGCGAAACCTGACCGGCCTCGATCAGAAAGAAATTATCGAAAGCTACAAA
AACCTGATGGGTAAAATCATCGACTTTGTGGATATCCTCTCCAAACCCGAACGCATTACCCAAATCATCC
GTGACGAACTGGAAGAAATCAAAACCAACTATGGCGACGAACGCCGCAGCGAAATCAACCCGTTCGGCGG
CGACATTGCCGATGAAGACCTGATTCCGCAACGCGAAATGGTCGTGACCCTGACCCACGGCGGCTATATA
AAAACCCAGCCGACCACCGACTATCAGGCTCAGCGTCGCGGCGGGCGCGGCAAACAGGCGGCTGCCACCA
AAGACGAAGACTTTATCGAAACCCTGTTTGTTGCCAACACGCATGACTATTTGATGTGTTTTACCAACCT
CGGCAAGTGCCACTGGATTAAGGTTTACAAACTGCCCGAAGGCGGACGCAACAGCCGCGGCCGTCCGATT
AACAACGTCATCCAGCTGGAAGAAGGCGAAAAAGTCAGCGCGATTCTGGCAGTACGCGAGTTTCCCGAAG
ACCAATACGTCTTCTTCGCCACCGCGCAGGGAATGGTGAAAAAAGTCCAACTTTCCGCCTTTAAAAACGT
CCGCGCCCAAGGCATTAAAGCCATCGCACTCAAAGAAGGCGACTACCTCGTCGGCGCTGCGCAAACAGGC
GGTGCGGACGACATTATGTTGTTCTCCAACTTGGGCAAAGCCATCCGCTTCAACGAATACTGGGAAAAAT
CCGGCAACGACGAAGCGGAAGATGCCGACATCGAAACCGAGATTTCAGACGACCTCGAAGACGAAACCGC
CGACAACGAAAACACCCTGCCAAGCGGCAAAAACGGCGTGCGTCCGTCCGGTCGCGGCAGCGGCGGTTTG
CGCGGTATGCGCCTGCCTGCCGACGGCAAAATCGTCAGCCTGATTACCTTCGCCCCTGAAACCGAAGAAA
GCGGTTTGCAAGTTTTAACCGCCACCGCCAACGGATACGGAAAACGCACCCCGATTGCCGATTACAGCCG
CAAAAACAAAGGCGGGCAAGGCAGTATTGCCATTAACACCGGCGAGCGCAACGGCGATTTGGTCGCCGCA
ACCTTGGTCGGCGAAACCGACGATTTGATGCTGATTACCAGCGGCGGCGTGCTTATCCGTACCAAAGTCG
AACAAATCCGCGAAACCGGCCGCGCCGCAGCAGGCGTGAAACTGATTAACTTGGACGAAGGCGAAACCTT
GGTATCGCTGGAACGTGTTGCCGAAGACGAATCCGAACTCTCCGGCGCTTCTGTAATTTCCAATGTAACC
GAACCGGAAGCCGAGAACTGA

In vitro *growth measurements*

Mutant and parent bacteria were grown on solid media for 18 hours, and suspensions were made in gonococci broth media (GCB). Each bacterial suspension was passed through a 1.2 micron filter to remove aggregates, and the filtered suspensions were inoculated into 30 ml of fresh GCB containing 100 µg/ml of streptomycin and supplemented with co-carboxylase, ferric nitrate, and mM NaH_2CO_3 at the concentrations described (39). Cultures were adjusted to an initial optical density (A_{600} = 0.075) and incubated under agitation at 37˚C. Absorbance recordings were taken each hour, and the average time needed to reach an A_{600} of 1.0 was established. All growth curves were conducted in triplicate.

For assessing relative *in vitro* fitness, competition co-cultures were performed with each mutant and corresponding parent strain. Similar numbers of mutant and parent bacteria were inoculated into supplemented GCB, starting with an A_{600} of approximately 0.08. Cultures were incubated under agitation at 37˚C and 100 µl aliquots were quantitatively cultured onto selective (0.125 µg/ml ciprofloxacin) and non-selective solid media at time zero to determine the input ratio. One hundred microliter aliquots were also collected and cultured in the same manner every 2 hours until an A_{600} of 1.0 was achieved (approximately 8 hours) to determine the output ratios. A competitive index (CI) was established by dividing the ratio of mutant to parent CFU at each time point by the input ratio of mutant to parent CFU. CI values greater than 1 were interpreted as enhanced fitness of the mutant relative to parent; whereas, CI values less than 1 indicated reduced mutant fitness relative to parent.

Minimum Inhibitory Concentration (MIC) measurements

The MICs of ciprofloxacin (Cip) and erythromycin (Em) were established for mutant and parent strain bacteria using a standard agar dilution assay (23). Reported ranges of intermediate level resistance and high level resistance to ciprofloxacin were used in accordance with CDC guidelines (23). The MIC of polymyxin B (PxB) was also determined using a standard agar dilution assay. PxB concentrations ranging from 200 to 900 µg/ml were used to establish MICs for strains JD4 and MS11.

In vivo *competitive infections*

Diestrus-stage female BALB/c mice (approximately 8 weeks old, National Cancer Institute, NIH) and *cnlp*$^{-/-}$ mice in the BALB/c background of a similar age (USUHS breeding colony) were administered water-soluble 17-β-estradiol subcutaneously as described (80) to promote susceptibility to *N. gonorrhoeae* infection. Antibiotics (2.4 mg streptomycin and 0.4 mg of vancomycin) were injected intraperitoneally twice each day and 0.04 g of trimethoprim sulfate per 100 ml of drinking water was provided throughout the course of the experiment to minimize commensal flora. Mutant and parent *N. gonorrhoeae* strains were grown on solid media for 18 hours, and suspensions were passed through a 1.2 micron filter to minimize aggregates. Bacterial suspensions were adjusted to the same A_{600} value and mixed in a 1 to 1 ratio. Twenty microliters of the mixed suspensions were inoculated intravaginally into mice and the inoculum was also quantitatively cultured on non-selective (total CFU) and selective (0.125 µg/ml

ciprofloxacin) (mutant CFU) solid media to establish input bacteria ratios. Vaginal swabs were quantitatively cultured on days 1, 3, 5, and 7 post-inoculation similarly to determine the number of parent and mutant CFU recovered over time (output ratio). The competitive index was calculated for each mouse at each time point by dividing the number of mutant to parent CFU from each culture day (output ratio) by the number of input mutant to parent CFU (input ratio). CI values greater than 1 indicated enhanced fitness of the mutant relative to parent; whereas, CI values less than 1 indicated reduced mutant fitness relative to parent.

RNA sequencing

RNA sequencing was performed on *N. gonorrhoeae* strains FA19Sm^{R}, FA1090, MS11, AK1, JD1, and JD4. Total RNA was isolated using TRIzol (Invitrogen) from cells grown on solid media after 18 hour incubation at 37º C. Ribosomal RNA (both 23S and 16S) was depleted using a MICROBExpress™ Bacterial mRNA Enrichment Kit (Ambion) according to the manufacturer's instructions. Since approximately 80% of the total pool of RNA consists of rRNA and tRNA, this depletion step is necessary for enrichment of less abundant RNA species within the pool. Following RNA enrichment, first and second strand cDNA library generation was performed (Invitrogen) and all samples were qualified for purity (260/280 > 1.7) and quantity (at least 10 µg of total cDNA). All cDNA samples were sent to the Emory University Genomics Center for paired-end 100 base read deep sequencing using the Illumina platform. Raw data files

were received from the Emory University Genomics Center, and data analysis was carried out using the CLC Bio Genomics Workbench suite.

Deep sequencing data analysis

The CLC Bio Genomics Workbench software suite was used for analysis of RNA sequencing data. Data from the imported files were normalized, and all sequence reads were mapped to the FA1090 published genome (the only annotated genome at the time). The length, count, and percent GC content for each read was captured, and coverage statistics were used to assess the quality of the coverage to reference genome. RNA sequencing analysis was selected for mapping to the FA1090 reference sequence with annotations. The Default software mapping settings were used for analysis. These mapping settings included a minimum length fraction of 0.9, a minimum similarity fraction of 0.8, and a maximum of 10 hits. The prokaryote setting was selected, and the Reads Per Kilobase of transcript per Million mapped reads (RPKM) measure of expression was used to address fold change differences in transcript profiles between mutant and parent strain. Scrutiny of global alterations in the transcriptome profile of mutant vs. parent gonococci included heat mapping and cluster analysis applications.

The Broad Institute's GenePattern 2.0 software program was used for cluster analysis of RNA sequencing data. RPKM data was imported into GenePattern. Software filters were balanced for both upregulated and down-regulated genes. These filters included a 2.0 fold change cutoff and a p value ≤ 0.05 (67).

Antimicrobial peptide susceptibility

Assessment of bacterial susceptibility to cathelicidin-related antimicrobial peptide (CRAMP-38) was carried out in a microtiter plate assay. CRAMP-38 was synthesized by the Microchemical Facility of Emory University (75). Lyophilized CRAMP-38 was reconstituted to a concentration of 5 mg/ml, and working stocks of 4, 8, 16, 32, and 64 µg/ml were generated in a 1:2 dilution of GCB and ddH_2O (dGCB). Bacterial strains to be tested were cultured on solid growth media. After 18 hrs incubation following an initial passage, bacterial suspensions were passed through a 1.2 micron filter to remove bacterial aggregates and diluted 1:500 in dGCB to an A_{600} of 0.075. Approximately 10^6 CFUs of each bacterial suspension was incubated with the appropriate concentration of CRAMP for a total volume of 100 µl per well. Polypropylene microtiter plates were incubated for 55 minutes at 37˚ C, and two 10-fold serial dilutions were made in GCB containing 0.05% saponin. One hundred microliters from each well were cultured onto non-selective solid media and allowed to incubate for 24 hours. CFU were enumerated and results from each parent/mutant set and were compared. The number of CFU recovered from untreated controls was used to define 100% recovery; the number of bacteria recovered from wells with CRAMP was expressed as a percentage of total recovery.

H_2O_2 sensitivity assay

Sensitivity to hydrogen peroxide was conducted using an H_2O_2 saturated disk diffusion assay previously described (79). Briefly, 100 µl aliquots of bacterial suspensions of the mutant and parent strains were adjusted to an A_{600} of 0.1 and cultured on solid media in triplicate. Sterile paper disks saturated in one of three serially-diluted concentrations of H_2O_2 (1 µM, 10 µM, and 10 mM) were placed onto solid GC plates. The diameter of the zone of growth inhibition around each disk was measured and recorded in millimeters following 24 hour incubation at 37˚C.

Sensitivity to H_2O_2 was also assessed using a modified version of a previously described liquid culture method (82). This method was chosen as a more quantitative measure of results obtained with the disk diffusion method. Briefly, mutant and parent gonococci were cultured to mid-log phase in liquid broth, diluted 1:10 in GCB, and subsequently split into separate cultures; one flask of GCB was cultured in the presence of 5 mM H_2O_2, while the other flask served as a negative control. Following a 15 minute shaking incubation at 37˚C, catalase was added to each H_2O_2-containing flask to a final concentration of 10 µg/ml followed by a 30 minute incubation to allow degradation of existing H_2O_2. Following the 30 minute incubation, 100 µl of each culture were quantitatively cultured onto solid GC media and allowed to incubate at 37˚C for 24 hours. CFUs from each plate were enumerated, and the numbers of CFUs recovered for each mutant/parent set were compared.

Statistical analyses

H_2O_2 disk diffusion measurements were recorded in triplicate and statistical significance was assessed for differences between the inhibition zone diameters around MS11 and JD4 using an unpaired *t* test. To assess the statistical significance for any differences in the competitive index obtained in CRAMP knock-out mice relative to wild-type BALB/c mice, a one-sample *t* test was performed on log-transformed data. The log transformation of the data was carried out to ensure the *p* values obtained from the *t* test were consistent with the geometric mean and confidence intervals.

Animal Use Assurances

Animal experiments were conducted in the laboratory animal facility at Uniformed Services University, which is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, under a protocol approved by the University's Institutional Animal Care and Use Committee

CHAPTER 3: Results

A gyrA *allele containing Ser91Phe and Asp95Asn substitutions confers intermediate level ciprofloxacin resistance to two other gonococcal strains*

We previously demonstrated that the Cip^I $gyrA_{91/95}$ resistant determinant in *N. gonorrhoeae* (AK1) provides an *in vivo* fitness advantage when competed with the Cip^S parent strain FA19 in the female mouse model of gonococcal genital tract infection. We moved this mutant allele into two other strains, FA1090 and MS11, to investigate the hypotheses that the elevated *in vivo* fitness associated with AK1 is not dependent on additional determinants and that mutant GyrA is sufficient to drive the observed fitness phenotype regardless of the strain background. The resultant mutants, JD1 and JD4, respectively, showed increased resistance to ciprofloxacin that fell within the Cip^I range (**Table 1**).

Table 3. Characteristics of the RNA sequencing analysis for each strain.

	FA1090	JD1	FA19	AK1	MS11	JD4
Total Reference Length	1,714,469	1,714,469	1,714,469	1,714,469	1,714,469	1,714,469
% GC Content	54	54	54	54	54	54
Total Read Count	12,541,110	7,367,320	10,299,940	5,428,116	9,660,509	3,689,000
Fraction of Reference Covered	0.99	0.99	0.97	0.98	0.98	0.98
Total Consensus Length	1,699,840	1,697,929	1,662,820	1,679,174	1,677,548	1,669,272

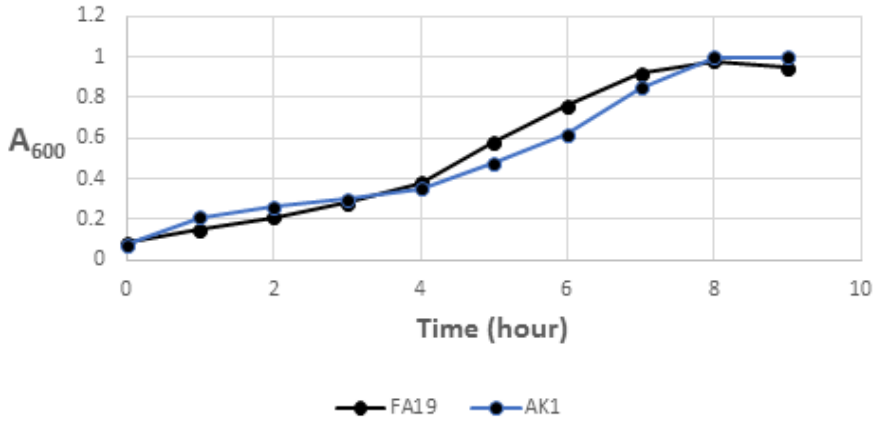
Growth measurements and* in vivo *competitive infections

The *gyrA*$_{91/95}$ mutants AK1, JD1, and JD4 displayed no significant difference in growth kinetics when cultured in liquid media compared to their isogenic parent strains (**Fig 5**). We also observed no difference in growth between JD1 and JD4 relative to that of their respective parent strains when co-cultured with their parent strains (**Fig. 6**). Following the observation that *gyrA*$_{91/95}$ mutant AK1 exhibits an *in vivo* fitness advantage during experimental murine infection, we hypothesized that the resistance-conferring mutations in *gyrA*$_{91/95}$ alone are sufficient to drive enhanced *in vivo* fitness of other gonococcal strains. Alternatively, the effect of the *gyrA*$_{91/95}$ mutations may be strain-specific as was reported for *gyrA* mutations in *C. jejuni* (51). Competitive murine infections were performed with each wild-type strain versus its respective *gyrA*$_{91/95}$ mutant. The results supported the hypothesis that the *gyrA*$_{91/95}$ allele is sufficient to drive an *in vivo* fitness advantage in different strain backgrounds. Interestingly, a difference in the degree of fitness advantage was observed with mutant JD4 displaying the most robust *in vivo* fitness advantage relative to parent strain MS11, followed by JD1 vs. FA1090 and AK1 vs. FA19 (**Fig. 7**)

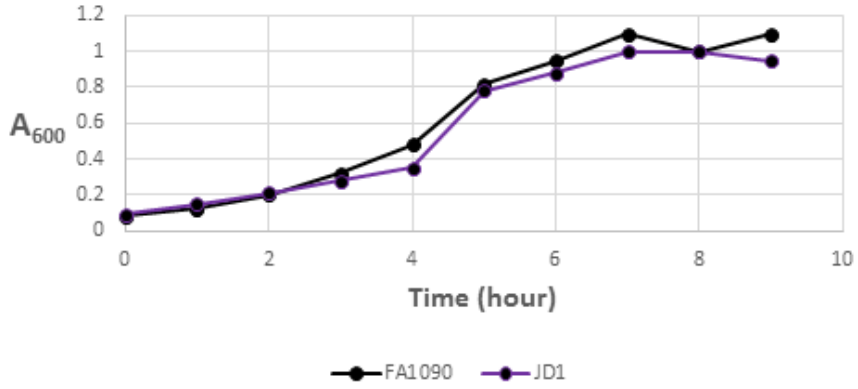
Figure 5. Growth kinetics of each *gyrA*$_{91/95}$ mutant versus its isogenic parent strain when cultured independently in liquid medium.

Growth was measured as change in absorbance at 600 nm over time. (A) FA19 versus AK1; (B) FA1090 versus JD1; (C) MS11 versus JD4.

A


1.2
1
0.8
0.6
0.4
0.2
0
A600
0
2
4
6
8
10
Time (hour)
FA19
AK1

B


1.2
1
0.8
0.6
0.4
0.2
0
A600
0
2
4
6
8
10
Time (hour)
FA1090
JD1

C

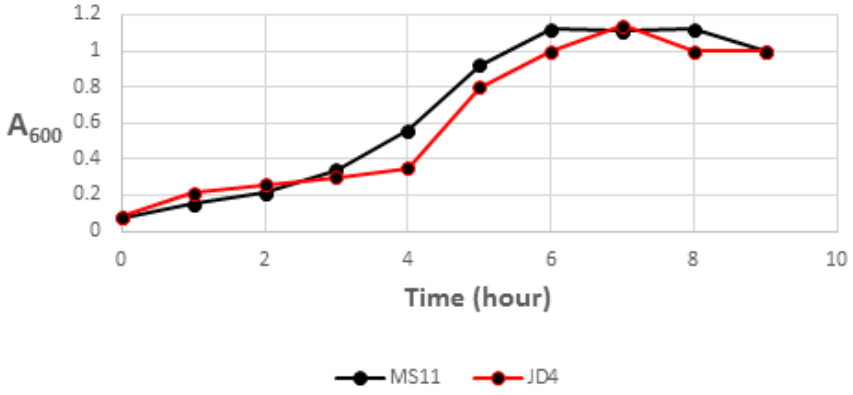

1.2
1
0.8
0.6
0.4
0.2
0
A600
0
2
4
6
8
10
Time (hour)
MS11
JD4

Figure 6. *In vitro* co-cultures of *gyrA*$_{91/95}$ mutant and wild-type parent strains.

Data are shown as competitive indices (CI) measured at time 0 and early, mid-logarithmic, late logarithmic and stationary phases. (A) FA1090 versus JD1; (B) MS11 versus JD4. *gyrA*$_{91/95}$ mutant AK1 was previously reported to be slightly attenuated relative to FA19 during co-culture (47) and FA19 and AK1 were not re-tested here.

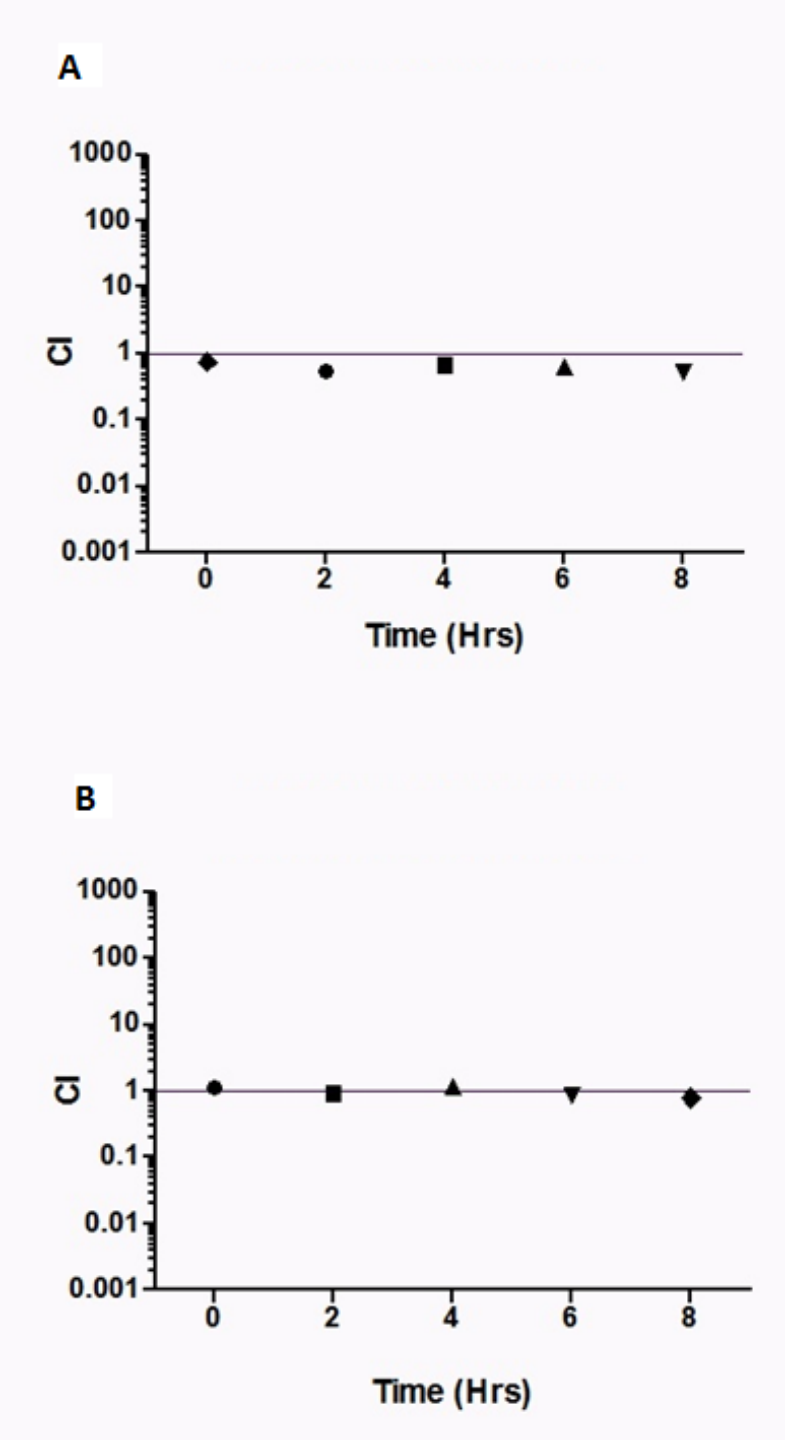
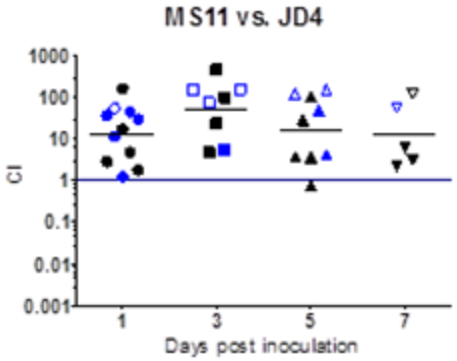
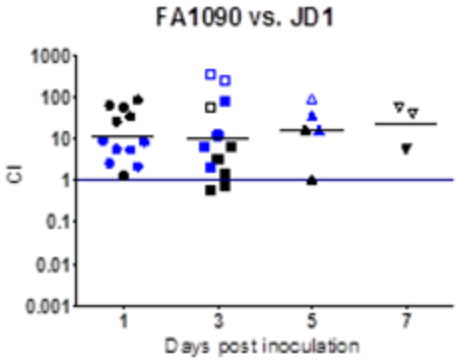

A
1000
100
10
1
0.1
0.01
0.001
CI
0
2
4
6
8
Time (Hrs)
B
1000
100
10
1
0.1
0.01
0.001
CI
0
2
4
6
8
Time (Hrs)

Figure 7. Three different *gyrA*$_{91/95}$ Cip^{I} mutants out-compete their Cip^{S} wild-type parents in an *in vivo* infection model.

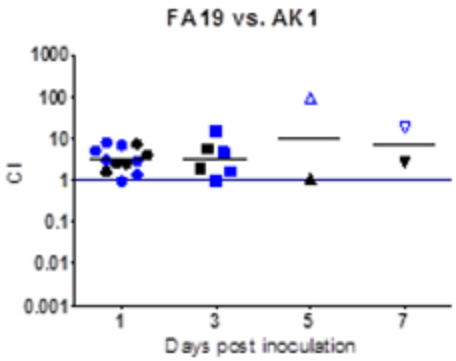
The geometric means of the CIs (GM CI) of the combined data are indicated by the horizontal bar and stated below each graph. Open symbols represent vaginal cultures that yielded only mutant bacteria and the limit of detection (10 CFU) was used as the number of wild-type bacteria recovered for these data points. The experiment was performed twice with black and blue data points corresponding to the first and second experiment, respectively.



GM CI: 12 - 51



GM CI: 10 - 24



GM CI: 3 - 10

Total RNA sequencing

As mentioned previously, DNA gyrase helps manage genomic topology during DNA replication, transcription and translation through the introduction of negative supercoiling. DNA gyrase facilitates negative superhelicity, in part, to help relax the stress introduced through local unwinding of the duplex at the sites of transcription (90). Alteration of genomic supercoiling has been suggested to influence the expression of some genes to such an extent that regional supercoiling is considered a mechanism of gene regulation (62; 69). Considering this activity, we hypothesized that the double point mutations in *gyrA* that are responsible for conferring an intermediate-level resistance to ciprofloxacin recondition the genome of *N. gonorrhoeae* to cause changes in the transcriptome that promote survival in the context of the host environment.

As mentioned previously, FA1090 was used as a reference for all strains and mutants used in this experiment, and CLC Bio was used to generate total read counts and differential expression values for each pair. A summary of each profile is listed in **Table 3**. A heat map analysis of the data from the RNA sequencing analysis clearly showed conserved changes in gene expression patterns in bacteria carrying the mutated $gyrA_{91/95}$ allele relative to parent strains (**Fig. 8**), an observation which is consistent with the mutated GyrA molecule affecting gene transcription as hypothesized. However, this analysis was carried out using balanced filters for positive and negative fold changes. When the fold change cutoff is set at ± 2, our cluster analysis produces only positive fold change differences for mutants

A number of interesting gene candidates with differential expression profiles were tentatively identified in each parent and mutant set. Since the enhanced fitness phenotype

was observed *in vivo* across three strains of *N. gonorrhoeae*, we chose to focus on transcript reads that were up-regulated (≥2-fold above for at least two of the three mutant/wild-type pairs) or down-regulated (≥1.5-fold below for at least two of the three mutant/wild type pairs) to identify genes that may be responsible for the observed *in vivo* phenotypes of the mutants. As shown in Fig. 5 a negative 2-fold change cutoff failed to produce down-regulated mutant genes; therefore, a decision was made to include a 1.5 fold decrease in mutant transcript reads to help enrich for relevant biological changes resulting from gene down-regulation. The total number of RNA sequence reads for parent strains was higher than that of mutant bacteria; because of the lower total mutant read count, we aimed for a negative fold change absolute value that was slightly lower (1.5) than the value set for the positive fold change cut-off (2). We acknowledge that many interesting genes may not have been captured as a result of these selection criteria; however, our goal was to identify phenotypically relevant transcriptional changes in an initial analysis with expanded data analysis planned for future studies.

These selection criteria produced a collection of 72 up-regulated genes in the mutant bacteria compared to their isogenic parent strains. In an effort to isolate a network or pathway of genes that might be responsible for enhanced *in vivo* fitness, annotated genes that were differentially altered in each set were grouped into five categories. These categories included genes with metabolic functions, genes encoding surface-expressed proteins, genes encoding transport-associated proteins, regulatory genes, and genes involved in iron uptake and utilization **(Tables 4, 5, 6 and 7).** A total of 26 genes were down-regulated; however, these reads were not assigned to a particular category (**Table 8**).

Many of the mutant sequence reads displaying the largest differential relative to isogenic parent bacteria had minimal or no annotation in the National Center for Biotechnology Information database, and only a putative functional domain existed for these candidates at the time of analysis. Up-regulated genes were selected based on the rationale that increased production a specific gene product could be responsible for promoting fitness within resource-limited environment of the host. For instance, expression of NGO2093 (a putative ferric receptor) was elevated greater than 2.5 fold in both JD4 and JD1 and nearly 2 fold in AK1 **(Table 7**). While iron availability is plentiful under *in vitro* culture conditions, sources of adequate iron during infection require bacterial scavenging of host-derived iron. Increased expression of surface factors that facilitate acquisition of host iron may provide a competitive edge for mutant bacteria over parent strains. Increased expression of genes involved in DNA damage and repair (**Table 6**) may offer mutant bacteria a means of sustained survival during oxidative stress.

Differential expression of genes involved in the biosynthesis of thiamine emerged as particularly interesting candidates responsible for enhanced fitness because thiamine, an essential vitamin or growth cofactor, is not produced by the host and therefore, increased production of thiamine in the mutants might confer a growth advantage during infection (**Table 4**). With a focus on the role that elevated thiamine biosynthesis potentially may contribute to *in vivo* fitness, we explored the hypothesis that mutant bacteria would outcompete parent strains *in vitro* in the absence of media supplementation with saturating thiamine.

Table 4. Up-regulated genes associated with the biosynthesis of thiamine.

Gene	Protein name or predicted function	JD4/MS11	JD1/FA1090	AK1/FA19
NGO2041	ThiC	6.67	2.99	1
NGO2003	Thiazole biosynthesis	2.62	2.23	1.45
NGO2006	ThiS	5.67	3.13	0.81
NGO2007	Thiamine-phosphate phosphorylase (ThiE)	2.89	4.26	1.58
NGO2005	ThiG	5.06	2.38	2.03
NGO2008	Oxidoreductase	6.02	3.15	1.76
NGO2009	Permease (sodium:solute symporter family)	2.34	2.38	1.06

Table 5. Up-regulated putative genes involved in gene regulation

Gene	Protein name or predicted function	JD4/MS11	JD1/FA1090	AK1/FA19
NGO0025	AraC family transcriptional regulator	6.16	2.65	1.89
nrdR	Transcriptional regulator	3.71	2.39	1.27
NGO0727	putative regulatory protein, gntR family	9.6	2.03	1.84

Table 6. Up-regulated genes associated with general metabolism and DNA damage and repair.

Gene	Protein name or predicted function	JD4/MS11	JD1/FA1090	AK1/FA19
NGO1521	Predicted acetate kinase	8.73	3.21	2.71
NGO0806	TCysN_NoDQ_II:	5.63	3.68	3.29
NGO0087	glycosyltransferase	2.62	2.05	1.78
NGO0031	Acetyltransferase; NAT (N-Acyltransferase)	2.17	2.02	1.96
NGO2008	Oxidoreductase	6.02	3.15	1.76
NGO0886	acyl-CoA dehydrogenase:	2.31	2.73	1.53
NGO0886	Acyl-CoA dehydrogenase	2.31	2.73	1.53
NGO1089	Possible arginine decarboxylase	2.16	2.58	4.95
NGO1091	Predicted binding partner of NGO1089	2.01	4.63	3.16
NGO0499	Putative ubiquinol-cytochrome C reductase	5	4.6	6.19
NGO0501	Predicted association with NGO0499	4.36	2.87	9.22
NGO0502	Predicted association with NGO499	20.43	2.67	5.67
NGO0555	Putative Zn-dependent protease	1.5	4.38	2.41
NGO1094	Putative Zn metallopeptidases	4.25	3.66	2.34
NGO1093	Putative PE family	5.47	4.33	2.69
NGO0518	NlpC/P60 superfamily	1.71	3.78	2.69
NGO1733	YjeQ	2.43	3.39	1.73
NGO0083	UDP-Glcnac inverting 4,6-dehydratase	3.05	3.29	1.78
NGO0026	Prolyl endopeptidase	1.41	3.13	2.01
NGO0076	Esterases	5.99	1.92	3.48
NGO0234	Coproporphyrinogen III oxidase	2.11	2.06	1.57
NGO0084	PglC	2.95	2.26	1.69
NGO1020	Putative integrase	2.39	1.62	2.27
NGO1136	Glycosyltransferase	2.62	5.11	1
NGO1739	Putative type II restriction endonuclease	8.73	2.83	1.08
NGO2047	FAD binding domain	2.27	2.27	1.04
NGO2068	Zinc metallopeptidase	3.09	2.09	1.23
NGO2050	Imelysin peptidase	2.53	2.81	1.08

Table 7. Up-regulated genes associated with iron utilization or transport.

Gene	Protein name or predicted function	JD4/MS11	JD1/FA1090	AK1/FA19
NGO0023	ABC transporter periplasmic binding protein	3.51	2.7	1.78
NGO2093	Fet A (ferric receptor)	2.63	2.92	1.99
NGO2092	Ferric periplasmic binding protein	1.66	3.08	2
NGO2011	Amino acid ABC transporter permease	1.17	3.83	1.39
NGO0500	Homology to Protein gp6; possible adhesion domain	5.24	3.4	4.29
NGO2091	ABC transporter; Transmembrane subunit	5.15	3.33	1.49
NGO2009	Permease (sodium:solute symporter family)	2.34	2.38	1.06
NGO2090	ABC transcporter permease	2.13	2.67	2.06
NGO0498	Pertactin-like passenger domains	2.89	2.97	4.31
NGO1097	Homology to FimV, Tfp pilus assembly	1.93	2.02	2.58
NGO2097	Prokaryotic membrane lipoprotein lipid attachment	2.37	2.27	1.34
NGO2092	Ferric enterobactin periplasmic binding protein	1.66	3.08	2
NGO0023	ABC transporter periplasmic binding protein	3.51	2.7	1.78
NGO1497	Transferrin receptor for transferrin utilization	5.2	6.81	No change

Table 8. Down-regulated genes.

Gene	Predicted Function	JD4/MS11	JD1/FA1090	AK1/FA19
NGO0589	Uracil-xanthine permease (*ncs2*)	-2.13	-2.13	-1.39
NGO0201	IS1016 transposase	-2.03	-1.59	-1.07
NGO0847	Predicted metal-binding protein related SecA	-2.01	-1.56	1.05
NGO1435	CadB	-2.01	-1.24	-1.59
NGO0631	Predicted functional association with GyrA	-1.62	-3.05	-1.34
NGO0629	GyrA	-1.54	-2.19	-1.11
NGO1671	Dephospho-coenzyme A kinase (DPCK)	-1.51	-2.77	-1.54
NGO1054	Predicted functional association with RecX	-1.24	-2.55	-1.63

Figure 8. Heat signatures for comparative transcriptome expression profiles.

Parent strain vs. mutant heat maps. The color scale differentiates lower levels of normalized transcript read counts (blue) from higher levels (red). The cluster shown reflects commonly up-regulated mutant genes relative to all three parent strains.


MS11
FA19
FA1090
JD4
AK1
JD1
Description
phage associated protein
hypothetical protein
hypothetical protein
hypothetical protein
phage associated protein
hypothetical protein
hypothetical protein
thiC
hypothetical protein
hypothetical protein
pseudo
pilin glycosylation protein
"ABC transporter permease, enterobactin"
hydrolase
hypothetical protein
phnA
phage associated protein
hypothetical protein
acetate kinase
ABC-type multidrug transport system
NGO0026
pilin glycosylation protein
phage associated protein
hypothetical protein
phage associated protein
hypothetical protein
hypothetical protein
acyl-CoA dehydrogenase
phage associated protein
phage associated protein
thiG
fetA
ABC transporter substrate-binding protein
phage associated protein
phage associated protein
hypothetical protein
phage associated protein
diaminohydroxyphosphoribosylaminopyrimidine deaminase
mpeR
hypothetical protein
phage associated protein
oxidoreductase
hypothetical protein
thiamin-phosphate pyrophosphorylase
ferric enterobactin periplasmic binding protein
ribosome-associated GTPase
phage associated protein
Pseudo
phage associated protein
phage associated protein
phage associated protein
acetyltransferase

In vitro growth in the absence of co-carboxylase

In the early 1940's, studies exploring the ideal *in vitro* growth conditions for *N. gonorrhoeae* identified a need to supplement culture media with co-carboxylase to be able to isolate occasional strains that were shown to be thiamine auxotrophs (48). The terminal product in the thiamine biosynthesis pathway is thiamine pyrophosphate; co-carboxylase is the common name. We hypothesized that the possible increased bio-production of thiamine in mutant bacteria, as suggested by the RNA sequencing data, would provide an *in vitro* growth advantage relative to parent strains when cultured in media without supplemented thiamine. However, mutant and parent *N. gonorrhoeae* grown independently or as co-cultures in liquid media not containing co-carboxylase supplementation displayed no difference in growth characteristics (data not shown). We therefore did not pursue this hypothesis further. We also considered the possibility that one of the genes shown to be up-regulated in our dataset (NGO2008) is an oxidoreductase that may provide decreased susceptibility to antimicrobial peptides (65).

Attempts to validate the elevated expression of selected transcripts that appeared to be differentially regulated (i.e. NGO 2008, *thiC*, and *thiG*) using quantitative real-time PCR were unsuccessful. It is possible that the low copy number of these transcripts limited the utility of quantitative PCR as a tool for validation of these candidates. Therefore, we directed our efforts towards *in vitro* assays designed to measure gonococcal resistance to host innate defenses.

Sensitivity to H_2O_2

Hydrogen peroxide disk diffusion assays were used as a surrogate assessment of sensitivity to oxidative stress *in vivo*. Only the *gyrA*$_{91/95}$ mutant in the MS11 background (mutant JD4) displayed a significantly smaller zone of inhibition around H_2O_2-saturated filter disks compared to the parent strain (**Fig. 9**). In an attempt to better quantify the sensitivity of the strains to H_2O_2, a broth culture method was also used. Bacteria were exposed to H_2O_2 in liquid culture under agitation in duplicate experiments, but none of the three mutants showed any difference in susceptibility to H_2O_2 relative to the respective parent bacteria.

Sensitivity to cationic antimicrobial peptides

Several cationic antimicrobial peptides are inducibly expressed by epithelial cells in the female genital tract, including the cathelicidins. These innate effectors are also carried within phagocytic granules. The *gyrA*$_{91/95}$ mutant JD4 was also more resistant to cathelicidin-related antimicrobial peptide (CRAMP), the murine homolog of the human cathelicidin LL-37, compared to parent strain MS11 as evidenced by a significantly higher effective concentration required for killing 50% of the bacterial population (EC_{50}) value (**Fig. 10**). This observed increase in CRAMP tolerance was supported with duplicate measurements of the minimum inhibitory concentration (MIC) of polymyxin B (PxB), which is a bacterial cationic antimicrobial peptides that is often used in *in vitro* studies. Agar dilution assays yielded a PxB MIC of 850 µg/ml against JD4, while the

PxB MIC for MS11 was 500 µg/ml. In contrast, and similar to results from H_2O_2 assays, the presence of the *gyrA*$_{91/95}$ mutations in the FA19 or FA1090 background did not confer statistically significant differences in susceptibility to CRAMP and PxB MIC were similar for mutant and parent strains.

In vivo competitive infection in wild-type and CRAMP$^{-/-}$ BALB/c mice

For an *in vivo* assessment of the susceptibility of MS11 and mutant JD4 to host antimicrobial peptides, we carried out parallel *in vivo* competition assays in wild-type BALB/c mice and BALB/c mice that are genetically deficient in the production of CRAMP. We predicted an ablation of the *in vivo* fitness advantage shown by JD4 when competed against MS11 in CRAMP-deficient BALB/c mice. Groups of wild-type and CRAMP-deficient mice were inoculated with mixed suspensions containing similar numbers of MS11 and JD4 bacteria, and the relative ratio of mutant to wild-type bacteria recovered from mice relative to that of the inoculum was determined as described in the Materials and Methods. In both sets of mice, mutant JD4 displayed a fitness advantage over MS11within one day of infection, with pure cultures of *gyrA*$_{91/95}$ mutant bacteria isolated from two mice at this time point (open circles) (Figure 10). On days 3 and 5, a 6 and 18 fold increase in fitness was observed for the mutant in normal BALB/c mice. In contrast, no fitness advantage was observed for JD4 in the CRAMP-deficient BALB/c mice. Comparison of the CIs obtained from wild-type and CRAMP-deficient mice on days 3 and 5 showed a significant difference at the level of $p = 0.02$ and $p = 0.013$ respectively. We conclude that the ciprofloxacin resistance-conferring *gyrA*$_{91/95}$ mutant of

strain MS11 demonstrates enhanced fitness in a surrogate model of female genital tract infection due to reduced susceptibility to CRAMP.

Figure 9 The *gyrA*$_{91/95}$ mutant in the MS11 background displayed decreased sensitivity to hydrogen peroxide.

Statistical significance was measured using an unpaired *t* test on mutant and parent inhibition zone diameters.

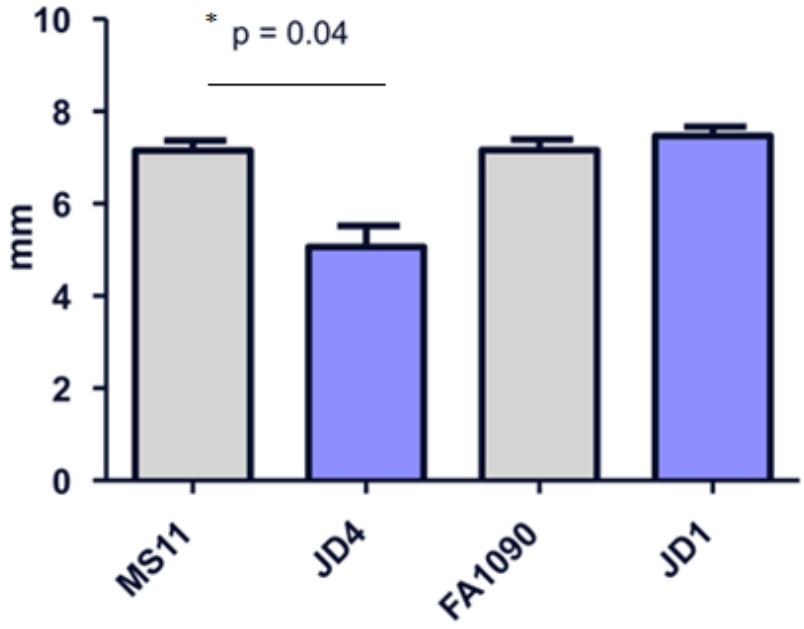

* p = 0.04
10
8
6
4
2
0
mm
MS11
JD4
FA1090
JD1

Figure 10. The *gyrA*$_{91/95}$ mutant in the MS11 background displayed decreased sensitivity to CRAMP.

The effective concentration of peptide required to kill 50% of the bacterial population (EC_{50}) for mutant and parent bacteria was determined in duplicate. CFUs were enumerated from the 10^{-5} dilution series, and each parent/mutant set was compared. The total CFU count from each untreated control was used to determine 100% recovery, and the CFUs recovered for each strain at each concentration of CRAMP tested was reflected as a percentage of total recovery. An EC_{50} value was extrapolated from the EC_{50} shift curve, and statistical significance was determined using an unpaired *t*-test. Only JD4 displayed a statistically significant increase in the CRAMP concentration required for an EC_{50}.

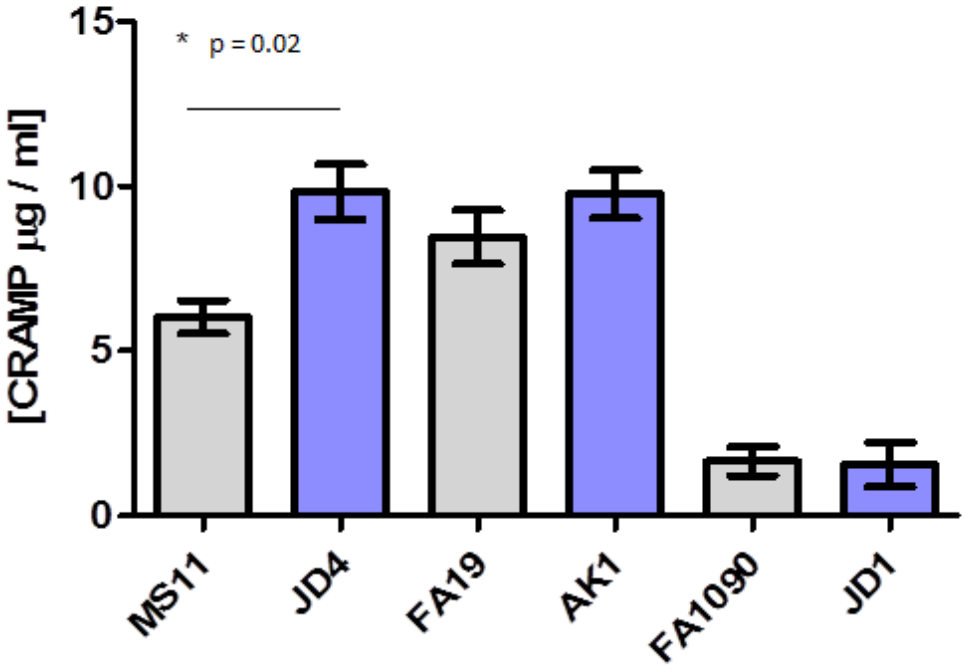
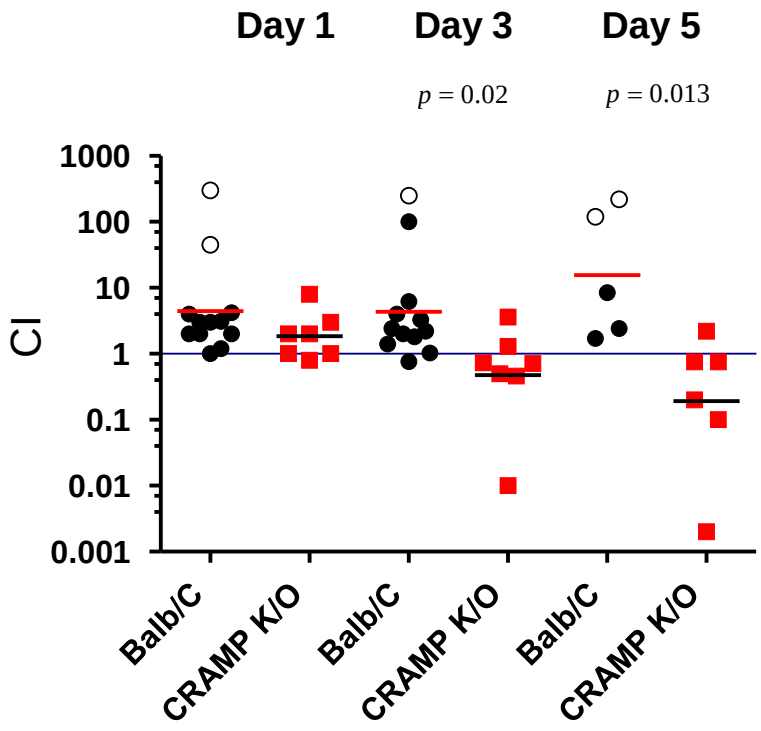

15
10
5
0
[CRAMP µg / ml]
* p = 0.02
MS11
JD4
FA19
AK1
FA1090
JD1

Figure 11. Competitive infections in CRAMP-sufficient (wild-type) and CRAMP-deficient BALB/c mice.

In vivo competitive infections competing $gyrA_{91/95}$ mutant JD4 against MS11 were conducted in parallel. The geometric mean CIs are shown as horizontal bars. Black symbols represent bacteria cultured from wild-type BALB/c mice, and red symbols represent bacteria cultured from CRAMP deficient mice. Open symbols represent vaginal cultures that yielded only mutant bacteria and the limit of detection (10 CFU) was used as the number of wild-type bacteria recovered for these data points. The experiment was performed twice and the combined data are shown. Comparison of the log-transformed data from days 3 and 5 showed a significant difference as indicated (unpaired *t* test).


Day 1
Day 3
Day 5
p = 0.02
p = 0.013
CI
1000
100
10
1
0.1
0.01
0.001
Balb/C
CRAMP K/O
Balb/C
CRAMP K/O
Balb/C
CRAMP K/O

CHAPTER 4: Discussion and Future Directions

There has been a steady rise of antibiotic resistance in *Neisseria gonorrhoeae* against several classes of antibiotics beginning with the introduction of sulfonamide therapy in the early 1940's. The loss of fluoroquinolone in 2007, and the more recent loss of the extended spectrum cephalosporins used as single therapies, has further complicated management of gonococcal infections. Current antibiotic treatment for gonorrhea is recommended to include a combined approach of ceftriaxone and azithromycin.

The era of fluoroquinolone treatment against gonorrhea exemplifies an accelerated evolutionary path to acquisition of resistance with less than 15-20 years between the introduction of fluoroquinolones against gonorrhea and the spread of an unacceptable percentage of resistant strains. The discovery that a commonly identified *gyrA* allele that confers intermediate level resistance to fluoroquinolones in *N. gonorrhoeae* also promotes *in vivo* fitness may help explain the short duration of the effectiveness of fluoroquinolone therapy. As previously mentioned, the acquisition of fluoroquinolone resistance in *N. gonorrhoeae* occurs through a step-wise process, where certain double point mutations in the *gyrA* gene produce two key amino acid changes at positions 91 and 95, which confers an intermediate level of resistance. An additional point mutation in the *parC* gene at position 86 confers high level resistance. While it appears that acquisition of both *gyrA* and *parC* mutations provides an unfavorable fitness cost *in vivo*, we hypothesize that the fitness advantage associated with *N. gonorrhoeae* harboring only the $gyrA_{91/95}$ resistance-conferring mutations facilitates the path to

fluoroquinolone resistance. It is important to note that enhanced fitness was not observed with *in vitro* co-culture experiments.

As was recently reported for strain FA19, here we showed that *gyrA*$_{91/95}$ mutations that confer intermediate-level FQ resistance result in enhanced *in vivo* fitness of *N. gonorrhoeae* in two other strain backgrounds, and the fitness advantage was greater in strains FA1090 and MS11 compared to strain FA19. This finding is different than that reported for *gyrA* mutants in *C. jejuni*, which only increased *in vivo* fitness in some *Campylobacter strains* (19). Since the introduction of *gyr*A$_{91/95}$ alone into three unrelated strains of *N. gonorrhoeae* is sufficient to promote fitness, we conclude that the influence of mutant *gyrA* alleles on the propagation and maintenance of antibiotic resistant *N. gonorrhoeae* may be stronger than that observed in *C. jejuni*.

Considering the ability of type II topoisomerases to provide broad regulation of the bacterial genome, we hypothesized that the fitness advantage associated with *gyrA*$_{91/95}$ mutations may be a consequence of an altered transcriptional profile, which could result in bacteria that are better suited for survival during genital tract infection. Differences were observed between three sets of mutant and parent transcriptomes, which supports our hypothesis and claims that resistance-conferring mutations in GyrA affect global levels of transcription in other Gram-negative bacteria (21). Although we categorized up-regulated and down-regulated genes with respect to phenotypes that may alter gonococcal fitness in an attempt to capture a molecular mechanism linked to our *in vivo* observations, we do not yet know which, if any of these differences are responsible for the observed *gyrA*$_{91/95}$-driven fitness advantage. In the context of infection, the resource-limited environment of the host pressures the ability of bacteria to synthesize or

scavenge essential nutrients or metabolites; additionally, increased expression of surface factors involved in evasion of innate defenses or adherence to host cells may be selected *in vivo*. Accordingly, genes we identified fall into those categories that may provide mutant bacteria with an advantage over parent, such as metabolism or iron uptake.

The use of *in vitro* grown rather than *in vivo* grown bacteria as the source of RNA for deep sequencing and the fact that the assay was done only once are two limitations to the RNA sequencing approach we utilized. While we see global changes in the transcriptome of the *gyrA* mutants relative to parent strains, capturing biologically relevant transcriptional changes that confer enhanced *in vivo* fitness may require bacterial growth within the host. Rather than sequencing RNA pools from bacteria grown *in vitro*, one could inoculate BALB/c mice with pure mutant or parent bacteria in triplicate, and on the day we typically see the largest competitive index (for example, day 3 from **Fig. 2**), we could inoculate a plate with vaginal material for culture and also transfer the vaginal swab directly into TRIzol® for immediate RNA extraction. Parallel sequencing of RNA from both the cultured *N. gonorrhoeae* as well as directly from the swab material, followed by subtraction of reads that do not map to the reference or cultured sequence should provide host-directed transcriptional profiles. There was not wide acceptance of this technique for defining the transcriptome of bacteria sampled directly from an infected host when we started this work; since the completion of this study in 2012, several methods have been employed to investigate host-directed RNA levels in bacteria (16; 29; 31; 42; 84; 99)

Considering the role that unidentified non-coding small RNAs may play in the regulation of key genes, a third limitation of the RNA seq analysis we performed is that

transcriptional differences in small RNAs might not have been detected. Key alterations may be limited by the degree of small non-coding RNA copy numbers, and potential quantitative differences in these regulatory RNAs may result in divergent protein expression between JD4 and MS11 for key genes that display relatively equal rates of transcription. Therefore, expanding subsequent rounds of deep sequencing to include an analysis of differences in small RNA levels would help broaden the spectrum of genes influenced by mutant GyrA.

Symptomatic gonorrhea infections present with a characteristic PMN influx at the site of infection. PMNs kill microbes through the production of reactive oxygen species and the release of granular contents, which include cationic antimicrobial peptides. Host-derived cationic antimicrobial peptides are also produced by epithelial cells and thus provide another barrier to bacterial colonization. We utilized *in vitro* assays to assess these challenges in an attempt to explain the fitness advantage associated with GyrA mutant bacteria. We found that the $gyrA_{91/95}$ mutant of strain MS11 was less susceptible to oxidative stress and antimicrobial peptides than the parent strain; however, this difference was not observed in the $gyrA_{91/95}$ mutants of FA19 or FA1090, although the concentration of CRAMP used against FA1090 may have been too high to detect a difference. While we have shown *in vitro* data suggesting that JD4 is less susceptible to H_2O_2 than MS11, we currently cannot further support the hypothesis that decreased susceptibility to oxidative stress contributes to enhanced *in vivo* fitness. PMN killing assays might support an *in vivo* role for the observed decrease in H_2O_2 susceptibility by JD4, although thus far, no known anti-oxidant factors, including catalase and cytochrome

C peroxidase produced by *N. gonorrhoeae* have been shown to protect the gonococcus from PMN killing *in vitro* or in NADPH-oxidase defective mice (39; 79; 103).

In contrast to our inability to comment on the relevance of H_2O_2 sensitivity *in vivo*, studies in CRAMP-deficient mice support $gyrA_{91/95}$-driven changes in resistance to CRAMP as the reason for the *in vivo* fitness advantage of mutant JD4. The mechanism by which $gyrA_{91/95}$ mutations confer increased resistance to antimicrobial peptides in strain MS11 is not known. We were surprised by the data reflecting this difference between JD4 and MS11, since MS11 is already highly resistant to cationic antimicrobial peptides due to a mutation that causes de-repression of the *mtrCDE* active efflux pump and a second mutation that generates an MtrR independent promoter (60; 98). Over-expression of the *mtrCDE* operon is one mechanism by which gonococci protect against antimicrobial peptides, but transcription of *mtrC* or *mtrA*, the pump activator, was not increased in mutant JD4 relative to the wild-type parent strain in the RNA seq analysis. However, it is still possible that the decreased sensitivity to CRAMP and polymyxin B exhibited by mutant JD4 may be the result of accelerated efflux through the MtrCDE system. Although the RNA sequencing data did not suggest altered expression of these pumps in the mutant bacteria, future attempts to assess transcriptional differences in *lptA* or *mtrCDE* using RT-PCR may yield different results. Additionally, one might predict that JD4 would be more resistant to other substrates of these efflux pumps such as progesterone and bile salts if efflux was increased. These possibilities should be explored.

Other mechanisms by which gonococci evade antimicrobial peptides is through changes in surface structures such as decoration of lipid A with phosphoethanolamine

(49) and sialylation of the N-neotetraose moiety of the LOS chain (Wu H and A E Jerse, in preparation). Both of these mechanisms are fully operable in MS11, and neither the phosphoethanolamine transferase (*lptA*) gene or sialyltranferase (*lst*) gene were up-regulated in mutant JD4 compared to the wild type strain. It is possible that *lptA* or *lst* expression is increased in the *gyrA* mutant bacteria indirectly through the altered expression of a non-coding small RNA that has not yet been identified. Alternatively, it is possible that the stability of LptA is enhanced in the *gyrA* mutants due to an elevated expression of oxidoreductase, which introduces stabilizing disulfide bonds to periplasmic proteins. *Neisseria spp.* express three oxidoreductases, and inactivation of each one these has been shown to increase sensitivity to antimicrobial peptides in *N. meningitidis* (66). Several genes with domains predicted to function as an oxidoreductase were shown to be elevated in the RNA sequencing data set (**Table 4 and 6**). The oxidoreductase with the greatest fold change difference relative to parental strains (NGO2008) was investigated for a potential role in increased resistance to polymyxin b. However, disrupting the NGO2008 open reading frame with a chloramphenicol-resistance cassette in both JD4 and MS11 did not alter the MIC to polymyxin b relative to unaltered mutant and parent bacteria. Furthermore, initial attempts to validate the NGO2008 transcriptional differences observed between mutant and parent in the RNA sequencing data with quantitative real-time PCR failed to suggest a difference in these transcript levels, albeit the RNA sequencing needs to be repeated and analyzed with more scrutiny.

It is interesting that $gyrA_{91/95}$ promotes *in vivo* fitness in all three strains of *N. gonorrhoeae* tested, but the mechanism responsible for this phenotype appears to be strain specific. This finding suggests that transcriptional changes directed by mutant

GyrA molecules can be tailored to the genetic background of each strain. This possibility adds to the value of repeating the transcriptional studies for mutant and parent bacteria. Alternatively, it is possible the mechanism responsible for enhanced fitness is the same in all three backgrounds examined. We observe a gradient in the promotion of *in vivo* fitness, with AK1 displaying the least advantage over parent followed by JD1 and JD4. This gradient is also reflected in many of the shared transcriptional changes captured in the RNA sequencing data. It is possible that decreases in sensitivity to antimicrobial peptides and oxidative stress in JD1 and AK1 are subtle enough to miss using *in vitro* measures of assessment, but substantial enough to provide a competitive edge over parent bacteria when the competition continues over days. Because of this possibility, conducting parallel competitive infections between JD1, AK1 and the respective parent strains in wild-type BALB/c and CRAMP deficient BALB/c mice may provide value.

Why a fluoroquinolone resistance-conferring *gyrA* mutant is able to enhance *in vivo* fitness across multiple strains of *N. gonorrhoeae* is an interesting question. Having evolved as a well-adapted human-specific pathogen, *N. gonorrhoeae* has acquired genomic determinants that are best suited for *in vivo* fitness. If the $gyrA_{91/95}$ allele provides the gonococcus with a survival advantage over the defined wild-type *gyrA*, nature should have selected the seemingly more fit $gyrA_{91/95}$ during this organism's evolution. It is possible that the *in vivo* advantage we see in our experimental murine model of infection is limited to state of an established infection. The $gyrA_{91/95}$ mutants may have a transmission disadvantage relative to the wild-type *gyrA* gonococcus, which would explain the apparent lack of selection for the $gyrA_{91/95}$ allele.

Another possibility is that the *gyrA* allele that we call wild-type is actually a compensatory mutation selected for as a compromise at some point after the antibiotic era ensued. As mentioned previously, antibiotic therapy for gonococcal disease began with the administration of sulfa drugs in the 1930's. In the decades that followed, the gonococcus was challenged with several classes of antibiotics and multiple generations within some of these classes. It is possible $gyrA_{91/95}$ is the true wild-type allele, and the gonococcus acquired the *gyrA* allele we currently refer to as "wild-type" at some point following decades of antibiotic exposure. Adaptation to treatment in the human host through the acquisition of antibiotic resistance determinants over time may have influenced the selection of a new *gyrA* allele best suited for evolving fitness requirements during the era of antibiotic pressure. With this hypothesis, we would expect $gyrA_{91/95}$ to be the dominant allele encountered in the gonococcus during the pre-antibiotic era, and remaining $gyrA_{91/95}$ alleles in the post-antibiotic era would contain fewer resistance determinants to additional classes of antibiotics. One interesting study consistent with this idea involved a 1998 assessment of intercity gonoccocal isolates within the United States displaying decreased susceptibility to fluoroquinolones. With a total of 783 clinical isolates from gonorrhea patients in five cities, Kilmarx and colleagues found that all of the isolates displaying decreased susceptibility to ciprofloxacin were β-lactamase negative and susceptible to spectinomycin, ceftriaxone, and cefixime (44). It is possible that these isolates did not contain an evolved *gyrA* providing decreased susceptibility to fluoroquinolones, but rather an allele was not as evolved as other gonococcal isolates containing a greater number of resistance determinants. It would be interesting to investigate the possibility that $gyrA_{91/95}$ is less fit than what we currently refer to as "wild-

type” *gryA* if these isolates contained some combination of other classes of resistance determinants.

In summary, these experiments suggest that in one strain of *N. gonorrhoeae*, an increased degree of tolerance to host defenses is driven by a resistance-conferring mutant GyrA allele, which may help explain the rapid proliferation of FQ-resistant *N. gonorrhoeae*; Future efforts should help elucidate the mechanism by which mutant JD4 is more resistant to host-produced CAMPs, which may be novel, as well as identify the basis of $gyrA_{91/95}$-driven in other gonococcal strains.

Appendix: The Impact of Fluoroquinolone Resistance-conferring *gyrA* and *parC* Mutations on Fitness and the Role of Compensatory Mutations

Background

As mentioned previously, antibiotic resistance provides a selective advantage for bacteria grown in the presence of antibiotic pressure, yet bacteria that contain antibiotic resistance mechanisms often carry the burden of reduced fitness relative to antibiotic susceptible parent strains (3; 7; 17; 58) . Many of the fitness costs associated with antibiotic resistance are accompanied by additional energy requirements needed for overexpression of genes involved in resistance, or the maintenance of extra-chromosomal elements responsible for conferring resistance. Compensatory mutations, either within the genes that confer resistance or in genes that are unrelated to resistance, can provide bacteria with adaptive means of overcoming fitness constraints associated with resistance to antibiotics (4; 46; 47; 58; 70).

In this appendix, I describe the work I have done to investigate the occurrence of compensatory mutations that restore fitness to gonococci possessing multiple antibiotic resistance mutations, specifically $gyrA_{91/95}$ and $parC_{86}$ mutations that confer high level ciprofloxacin resistance and *mtr* locus mutations that confer resistance to macrolide antibiotics and high level penicillin.

In our work with *gyrA* and *parC* mutations, we found that a strain with an *mtrR* promoter mutation ($mtrR_{-79}$) and the first and second-step type 2 topoisomerase mutations in *gyrA* and *parC* exhibited the greatest fitness cost with respect to growth *in vitro* and

recovery from experimentally infected mice. This mutant, AK12, was derived from mutant AK11 (*gyrA*$_{91/95}$, *mtrR*$_{-79}$), and interestingly, a compensatory mutant of strain AK12 was selected during an *in vivo* competition experiment between mutants AK12 and AK11. This mutant, called AK13, was isolated on day 5 post-inoculation from a mouse that initially showed a significant drop in the total number of CFU recovered and in the recovery of AK12 relative to AK11, followed by a return of high numbers of gonococci, all of which were highly ciprofloxacin resistant. Mutant AK13 also showed a pronounced growth advantage compared to its parent AK12 and the wild type strain. Since MIC analysis of AK13 showed no difference in the resistance to ciprofloxacin compared to AK12, we hypothesized that the means of over-coming the fitness burden displayed by AK12 was not through repair of quinolone-conferring mutations, but rather through the acquisition of one or more compensatory mutations.

The parent of strains AK11, AK12, and AK13 is KH15, which is the parent strain FA19 containing an *mtrR*$_{-79}$ mutation. This mutation is a single base deletion in the inverted repeat sequence between the -10 and -35 regions of the *mtrR* promoter (36). This single base deletion in KH15, AK11, and AK12 disrupts the optimal spacing between the -10 and -35 elements required for RNA polymerase association and subsequent transcription. Therefore, *mtrR* is not efficiently transcribed in KH15, AK11, and AK12 allowing for over-expression of the *mtrCDE* efflux pump. Since our laboratory previously defined a fitness gradient for *mtrCDE* expression for different types of *mtr* locus mutations (97; 98), we hypothesized that the *mtrR* promoter region or structural gene in AK13 may contain additional mutations that help restore fitness. We also speculated that changes may be present in the QRDR region of AK13. Therefore,

we sequenced the intergenic region between mtrR and *mtrCDE* as well as the full-length *parC* and *gyrA* genes in strains FA19, AK12, and AK13 in an attempt to identify potential compensatory mutations that would help explain AK13's *in vivo* fitness enhancement. Sequence analysis of the *parC* gene showed no differences between mutants AK13 and AK12, and the presence of a D86N mutation compared to strain FA19 as expected. Similarly, the *gyrA* gene of AK12 contains a S91F and D95N amino acid changes relative to strain FA19 as expected. However, as shown in **Fig. 12**, AK13 carries a point mutation in the third nucleotide of codon 91 of the *gyrA* gene. Relative to parent AK12, this TTC → TTA mutation leads to a F91L substitution in the QRDR of *gyrA*. When we looked at the *mtrR* upstream sequence from AK13, we also saw an insertion mutation within the inverted repeat sequence of the promoter that restores the spacing of the -10 and -35 sequences needed for *mtrR* expression and natural repression of the *mtrCDE* operon. How the *mtrR* promoter insertion mutation or the F91L mutation in *gyrA* may lead to elevated bacterial fitness is not known. It is very possible that additional fitness-enhancing mutations exist in AK13, and whole genome sequencing is needed to explore this possibility.

While the mechanism by which the fitness of Cip^R mutant AK13 was restored is not yet known, we conclude that selection for compensatory mutations is a mechanism by which fitness can be restored in highly Cip^R strains. These data were published in the manuscript entitled "Impact of fluoroquinolone resistance mutations on gonococcal fitness and *in vivo* selection for compensatory mutations" (47). To continue to investigate the hypothesis that compensatory mutations may allow high Cip^R gonococci to be maintained in the absence of fluoroquinolone treatment, we introduced the second

step *parC*$_{86}$ mutation in *gyrA*$_{91/95}$ mutants of strains FA1090 and MS11 (mutants JD1 and JD4, respectively). We predicted that the resultant mutants, JD1.2 and JD4.2 would exhibit elevated ciprofloxacin resistance and an associated fitness cost. We also hypothesized that procurement of compensatory mutations in the fitness-burdened Cip^{R} mutants of *N. gonorrhoeae* will elevate *in vivo* fitness without sacrificing the magnitude of antibiotic resistance

Figure 12. Highlighted QRDR GyrA and parC amino acid sequences of Cip^S and Cip^R *Neisseria gonorrhoeae*

Cip^S *Neisseria gonorrhoeae* (FA19), Cip^R (AK12), and the Cip^R compensatory mutant (AK13) are shown at the top of the figure. Shown at the bottom is the intergenic promoter region of the *mtr*CDE efflux pump repressor (*mtr*R).

gyrA (QRDR)

							91				95						
	tac	cac	ccc	cac	ggc	gat	tcc	gca	gtt	tac	gac	acc	atc	gtc	ctg	atg	gcg
FA19	Y	H	P	H	G	D	S	A	V	Y	D	T	I	V	R	M	A
AK12	·	·	·	·	·	·	F	·	·	·	N	·	·	·	·	·	·
AK13	·	·	·	·	·	·	L	·	·	·	N	·	·	·	·	·	·

parC (QRDR)

								86									
	ggt	aaa	tac	cat	ccg	cac	ggc	gac	agt	tcc	gcc	tat	gag	gcg	atg	gtg	cgc
FA19	G	K	Y	H	P	H	G	D	S	S	A	Y	E	A	M	V	R
AK12	·	·	·	·	·	·	·	N	·	·	·	·	·	·	·	·	·
AK13	·	·	·	·	·	·	·	N	·	·	·	·	·	·	·	·	·

mtrR-mtrCDE Intergenic Region

										-10																			-35						
FA19	g	g	g	c	g	g	a	t	t	a	t	a	a	a	a	a	g	a	c	t	t	t	t	t	a	t	c	c	g	t	g	c	a	a	t	c	g	t
AK12	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	–	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
AK13	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·

Hypothesis 2:

Procurement of compensatory mutations in the fitness-burdened Cip^R mutants of *N. gonorrhoeae* will elevate *in vivo* fitness without sacrificing the magnitude of antibiotic resistance.

Materials and methods

Bacterial strains and mutant construction

Neisseria gonorrhoeae $gyrA_{91/95}$ mutants JD1 and JD4 were used as parents for generation of Cip^R bacteria containing the $parC_{86}$ allele that provides this elevated resistance. JD1.2 and JD4.2 $gyrA_{91/95}$ and $parC_{86}$ mutants were constructed by allelic exchange with donor PCR amplicon containing the Asp86Asn mutation. The method employed followed that previously reported for the construction of AK2 and AK12 (35; 47). Briefly, a 1.6-kb region of *parC* was amplified from AK2 using parC1-for and parC1-rev primers from **Table 2**. JD1 and JD4 were passed on non-selective solid media following an initial sub culture. Bacteria were suspended in PBS and adjusted to an A_{600} of 0.05. Ten microliters of the bacterial suspension were spotted onto solid non-selective GC media followed by direct overlay of approximately 1 µg of template DNA used for targeted homologous recombination. Following incubation at 37º C for 24 hours, individual colonies were isolated and streaked onto solid growth media containing 2µg/ml of ciprofloxacin for selection of $gyrA_{91/95}$ / $parC_{86}$ mutants. Freezer stocks were

generated, and mutant bacteria were sequenced for positive identification of the altered nucleotide sequence (**Figures 13** and **14**).

Figure 13. Open reading frame sequence of the *parC* sequence from JD1.2.

The highlighted codon shows the GAC → AAC point mutation that produces the Asp → Asn amino acid transition at position 86 (D86N).

ATGAATACGCAACCGCACGCTTCCCATACCGATTCCAACACGCTGATGCTCGGCCGATACGCCGAACGCGC
CTATCTCGAATACGCCATGAGCGTGGTCAAAGGCCGCGCGCTGCCTGAAGTTTCAGACGGCCAAAAGCCCG
TGCAGCGGCGCATTTTGTTTGCCATGCGCGATATGGGTTTGACGGCGGGGGCGAAGCCGGTGAAATCGGCG
CGCGTGGTCGGCGAGATTTTGGGTAAATACCATCCGCACGGCAACAGTTCCGCCTATGAGGCGATGGTGCG
CATGGCTCAGGATTTTACCTTGCGCTATCCCTTAATCGACGGCATCGGCAACTTCGGTTCGCGCGACGGCG
ACGGGGCGGCGGCGATGCGTTACACCGAAGCGCGGCTCACGCCGATTGCGGAATTGCTGTTGTCCGAAATC
AATCAGGGGACGGTGGATTTTATGCCGAACTACGACGGCGCGTTTGACGAGCCGCTGCACCTTCCCGCCCG
CTTGCCTATGGTGTTGCTCAACGGCGCGTCGGGCATCGCGGTGGGTATGGCGACCGAGATTCCGTCGCACA
ATTTGAACGAAGTCACGCAGGCGGCGATTGCACTGTTGAAGAAACCGACGCTGGAAACCGCCGACCTGATG
CAATATATTCCTGCTCCCGATTTTGCCGGCGGCGGTCAAATCATCACGCCGGCGGACGAATTGCGCCGTAT
TTACGAAACCGGCAAGGGCAGCGTGCGCGTGCGTGCGCGTTATGAAATCGAGAAATTGGCGCGCGGACAGT
GGCGCGTCATCGTAACCGAACTGCCGCCGAACGCCAATTCCGCCAAAATCCTTGCCGAAATCGAAGAGCAA
ACCAACCCGAAACCGAAAGCGGGCAAGAAGCAGCTCAACCAAGACCGGCTTAATACCAAAAAGCTGATGCT
GGATTTAATCGACCGCGTGCGCGACGAGTCCGACGGCGAACATCCCGTGCGCCTTGTATTTGAACCGAAAT
CCAGCCGCATCGATACCGATACCTTCATCAACACGCTGATGGCGCAAACTTCGCTGGAAGGCAATGTGTCC
ATGAACTTGGTGATGATGGGTTTGGACAACCGCCCCGCGCAGAAAAACCTGAAAACGATTTTGCAGGAATG
GCTGGATTTCCGCATCGTTACCGTAACACGCCGTCTGAAATTCCGTTTAAACCAAGTGGAAAAACGGCTGC
ACATCCTCGAAGGCCGTCTGAAAGTCTTTCTGCACATCGACGAAGTGATTAAAGTCATCCGCGAATCGGAC
GACCCGAAAGCCGATTTGATGGCGGTGTTCGGGCTGACCGAAATCCAGGCCGAAGACATTTTGGAAATCCG
CCTGCGTCAGTTGGCGCGTTTGGAAGGTTTCAAACTCGAAAAAGAATTGAACGAATTGCGCGAAGAACAAG
GCCGTCTGAATATCTTTTTGGGCGACGAAAACGAAAAACGCAAGCTGATTATCAAAGAGATGCAGGCGGAC
ATGAAGCAGTTCGGCGACGCGCGCCGCACGCTGGTGGAAGAGGCCGGACGCGCCGTGCTGACACAAACCGC
CGCCGACGAACCCATCACGCTGATTTTGTCGGAAAAAGGCTGGATACGCAGCCGTGCCGGACATAATCTCG
ATTTGAGCCAAACCGCGTTCAAAGAAGGCGACCGCCTCAAACAAACCCTTGAAGGCCGCACTGTTTTACCC
GTCGTCATCCTCGATTCATCGGGCAGAACCTACTCGATCGATGCCGCCGAAATCCCCGGCGGACGCGGCGA
CGGCGTACCGGTTTCCTCCTTAATCGAGTTGCAAAACGGCGCGAAACCCGTCGCGATGTTGACAGGATTGC
CGGAACAACATTATTTATTATCAAGCAGCGGCGGCTACGGCTTTATCGCCAAGCTGGGCGATATGGTCGGA
CGCGTGAAAGCGGGCAAAGTGGTGATGACCGCAGACAGCGGCGAAACCGTCCTGCCGCCGGTTGCCGTCTA
TGCCTCCTCGTTCATCAACCCCGACTGCAAAATCATTGCAGCCACCAGTCAAAACCGCGCCCTCGCCTTCC
CCATCGGCGAATTGAAAATTATGGCGAAAGGCAAAGGACTGCAAATCATCGGATTAAACGCCGGCGAATCG
ATGACGCATACCGCCGTTTCTTCCGAGCCGGAAATCCTGATTGAAAGCGAAGGCAGGCGCGGCGCGGCGCA
CAAAGACCGCCTCCCCGTCGCCCTGATTGAGGCAAAACGCGGCAAAAAAGGCAGACTGTTGCCCATATCGG
GCAGCCTGAAACAGCTTTCTTCCCCCAAATAA

Figure 14. Open reading frame sequence of the *parC* sequence from JD4.2.

The highlighted codon shows the GAC → AAC point mutation that produces the Asp → Asn amino acid transition at position 86 (D86N).

ATGAATACGCAACCGCACGCTTCCCATACCGATTCCAACACGCTGATGCTCGGCCGATACGCCGAACGCGC
CTATCTCGAATACGCCATGAGCGTGGTCAAAGGCCGCGCGCTGCCTGAAGTTTCAGACGGCCAAAAGCCCG
TGCAGCGGCGCATTTTGTTTGCCATGCGCGATATGGGTTTGACGGCGGGGGCGAAGCCGGTGAAATCGGCG
CGCGTGGTCGGCGAGATTTTGGGTAAATACCATCCGCACGGC**AAC**AGTTCCGCCTATGAGGCGATGGTGCG
CATGGCTCAGGATTTTACCTTGCGCTATCCCTTAATCGACGGCATCGGCAACTTCGGTTCGCGCGACGGCG
ACGGGGCGGCGGCGATGCGTTACACCGAAGCGCGGCTCACGCCGATTGCGGAATTGCTGTTGTCCGAAATC
AATCAGGGGACGGTGGATTTTATGCCGAACTACGACGGCGCGTTTGACGAGCCGCTGCACCTTCCCGCCCG
CTTGCCTATGGTGTTGCTCAACGGCGCGTCGGGCATCGCGGTGGGTATGGCGACCGAGATTCCGTCGCACA
ATTTGAACGAAGTCACGCAGGCGGCGATTGCACTGTTGAAGAAACCGACGCTGGAAACCGCCGACCTGATG
CAATATATTCCTGCTCCCGATTTTGCCGGCGGCGGTCAAATCATCACGCCGGCGGACGAATTGCGCCGTAT
TTACGAAACCGGCAAGGGCAGCGTGCGCGTGCGTGCGCGTTATGAAATCGAGAAATTGGCGCGCGGACAGT
GGCGCGTCATCGTAACCGAACTGCCGCCGAACGCCAATTCCGCCAAAATCCTTGCCGAAATCGAAGAGCAA
ACCAACCCGAAACCGAAAGCGGGCAAGAAGCAGCTCAACCAAGACCGGCTTAATACCAAAAAGCTGATGCT
GGATTTAATCGACCGCGTGCGCGACGAGTCCGACGGCGAACATCCCGTGCGCCTTGTATTTGAACCGAAAT
CCAGCCGCATCGATACCGATACCTTCATCAACACGCTGATGGCGCAAACTTCGCTGGAAGGCAATGTGTCC
ATGAACTTGGTGATGATGGGTTTGGACAACCGCCCCGCGCAGAAAAACCTGAAAACGATTTTGCAGGAATG
GCTGGATTTCCGCATCGTTACCGTAACACGCCGTCTGAAATTCCGTTTAAACCAAGTGGAAAAACGGCTGC
ACATCCTCGAAGGCCGTCTGAAAGTCTTTCTGCACATCGACGAAGTGATTAAAGTCATCCGCGAATCGGAC
GACCCGAAAGCCGATTTGATGGCGGTGTTCGGGCTGACCGAAATCCAGGCCGAAGACATTTTGGAAATCCG
CCTGCGTCAGTTGGCGCGTTTGGAAGGTTTCAAACTCGAAAAAGAATTGAACGAATTGCGCGAAGAACAAG
GCCGTCTGAATATCTTTTTGGGCGACGAAAACGAAAAACGCAAGCTGATTATCAAAGAGATGCAGGCGGAC
ATGAAGCAGTTCGGCGACGCGCGCCGCACGCTGGTGGAAGAGGCCGGACGCGCCGTGCTGACACAAACCGC
CGCCGACGAACCCATCACGCTGATTTTGTCGGAAAAAGGCTGGATACGCAGCCGTGCCGGACATAATCTCG
ATTTGAGCCAAACCGCGTTCAAAGAAGGCGACCGCCTCAAACAAACCCTTGAAGGCCGCACTGTTTTACCC
GTCGTCATCCTCGATTCATCGGGCAGAACCTACTCGATCGATGCCGCCGAAATCCCCGGCGGACGCGGCGA
CGGCGTACCGGTTTCCTCCTTAATCGAGTTGCAAAACGGCGCGAAACCCGTCGCGATGTTGACAGGATTGC
CGGAACAACATTATTTATTATCAAGCAGCGGCGGCTACGGCTTTATCGCCAAGCTGGGCGATATGGTCGGA
CGCGTGAAAGCGGGCAAAGTGGTGATGACCGCAGACAGCGGCGAAACCGTCCTGCCGCCGGTTGCCGTCTA
TGCCTCCTCGTTCATCAACCCCGACTGCAAAATCATTGCAGCCACCAGTCAAAACCGCGCCCTCGCCTTCC
CCATCGGCGAATTGAAAATTATGGCGAAAGGCAAAGGACTGCAAATCATCGGATTAAACGCCGGCGAATCG
ATGACGCATACCGCCGTTTCTTCCGAGCCGGAAATCCTGATTGAAAGCGAAGGCAGGCGCGGCGCGGCGCA
CAAAGACCGCCTCCCCGTCGCCCTGATTGAGGCAAAACGCGGCAAAAAAGGCAGACTGTTGCCCATATCGG
GCAGCCTGAAACAGCTTTCTTCCCCCAAATAA

In vitro *growth measurements*

Mutant and parent strain bacteria were grown on solid media for 18 hours, and suspensions were made in GCB. Each bacterial suspension was passed through a 1.2 micron filter to discourage aggregate formation, and the A_{600} was adjusted to 0.075 into 30 ml of GCB. Thirty milliliter cultures for each mutant and parent strain set were incubated under agitation at 37˚C. Optical density recordings were taken each hour, and the average time needed to reach an A_{600} of 1.0 was established. All growth curves were run in duplicate.

For assessing relative *in vitro* fitness, competition experiments were carried out using similar numbers of the mutant and parent strain being compared. Each mixed culture was initiated at an A_{600} of approximately 0.08 and 100 µl aliquots were plated onto selective (0.125 µg/ml ciprofloxacin) and non-selective solid media. Mutant to parent ratios were obtained by subtracting the CFUs on the selective plates from the CFUs on the non-selective plates. Subsequent 100 µl aliquots were collected and plated in the same manner every 2 hours until an A_{600} of 1.0 was achieved. A competitive index (CI) was established by dividing the ratio of mutant to parent CFUs recorded from the plates at each time point by the ratio of input mutant to parent CFUs. CI values greater than 1 illustrates enhanced fitness of the mutant relative to parent; whereas, CI values less than 1 illustrate depressed mutant fitness relative to parent.

MIC measurements

Minimum inhibitory concentrations (MICs) of ciprofloxacin were established for mutant and parent strain bacteria using a standard agar dilution assay (23). Reported ranges of intermediate level resistance and high level resistance to ciprofloxacin were reported in accordance with CDC guidelines.

In vivo *competitive infections*

BALB/c mice (approximately 8 weeks old) in the diestrus stage of the estrous cycle were administered 17-β-estradiol subcutaneously to optimize susceptibility to GC infection 2.4 mg streptomycin and 0.4 mg of vancomycin were injected intraperitoneally twice each day and 0.04 g of trimethoprim sulfate per 100 ml of drinking water was provided throughout the course of the experiment to minimize growth of commensal flora. Mutant and parent strain *N. gonorrhoeae* were grown on solid media for 18 hours, and suspensions were passed through a 1.2 micron filter to minimize aggregations. Mutant and parent strains were adjusted to equal A_{600} optical densities and mixed in a 1 to 1 ratio. Mixed 20 µl inocula were introduced intravaginally into mice, and each inoculum was plated on both non-selective and selective (0.125 µg/ml ciprofloxacin) solid media to establish input bacteria ratios. Vaginal swab cultures performed on days 1, 3, 5, and 7 served as output bacteria ratios. A competitive index was calculated by dividing the ratio of mutant to parent CFUs from each culture day by the ratio of input mutant to parent CFUs. CI values greater than 1 illustrates enhanced fitness of the mutant relative to parent; whereas, CI values less than 1 illustrate depressed mutant fitness relative to parent.

Results

To investigate the phenomenon of selection for compensatory mutations as a mechanism for restoring fitness in the context of ciprofloxacin resistant mutants, we first aimed to move the second-step Cip^R mutation in *parC* to strains JD1 and JD4. We named these strains JD1.2 and JD4.2 respectively. After establishing that JD1.2 and JD4.2 had the predicted the Cip^R phenotype (**Table 1**), we assessed *in vitro* growth by culturing each strain separately in liquid culture or together with its ciprofloxacin susceptible parent strain. While there appeared to be no difference between JD1.2 and FA1090 when cultured independently, surprisingly, mutant JD4.2 grew faster than wild-type MS11. JD4.2 also displayed greater *in vitro* fitness than MS11 during co-culture measurements (**Fig. 15**).

We next conducted competitive infections in the murine model to assess the relative *in vivo* fitness of JD1.2 to FA1090 and JD4.2 to MS11 (**Fig. 16**). Fitness studies with mutant JD1.2 supported our hypothesis that the Cip^R-conferring mutation in *parC* would reduce fitness relative to parental strains of *N. gonorrhoeae*. Cultures taken on days 3 and 5, all showed decreased fitness for JD1.2. However, in one mouse only Cip^R bacteria were recovered on Day 7 post inoculation, suggesting selection for a compensatory mutation had occurred during infection. This strain was isolated and frozen for further investigation.

In contrast to mutant JD1.2, mutant JD4.2 displayed a high competitive index relative to MS11 throughout the entire infection period. Based on the increased growth of JD1.2 *in vitro* relative to MS11 and the enhanced fitness of JD1.2 *in vivo*, we speculate that during the process of mutating the *parC* allele of JD4, a spontaneous mutation arose

elsewhere in the genome which resulted in elevated fitness. While this remains an interesting finding and should be pursued, the *parC* mutation should be reintroduced into JD4 for future studies on in-host selection for compensatory mutations in Cip^R MS11 mutant bacteria carrying *gyrA*, *parC* mutations.

Figure 15. Growth curves for MS11, FA1090, and the two Cip^R mutants

The two Cip^R mutants JD1.2 and JD4.2 are shown in panel A. Panels B and C show results of the in vitro co-cultures between JD4.2 vs. MS11 and JD1.2 vs. FA1090, respectively.

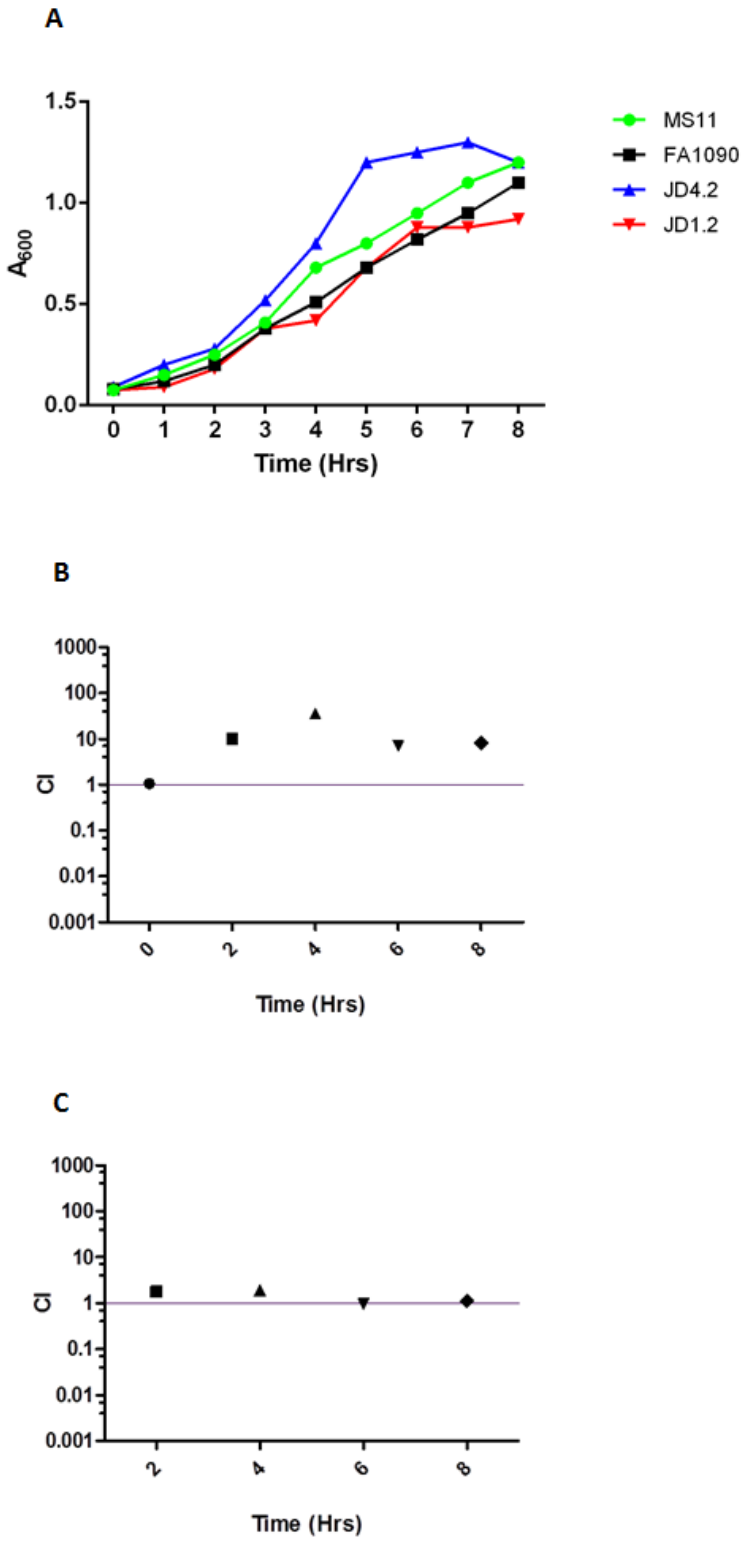
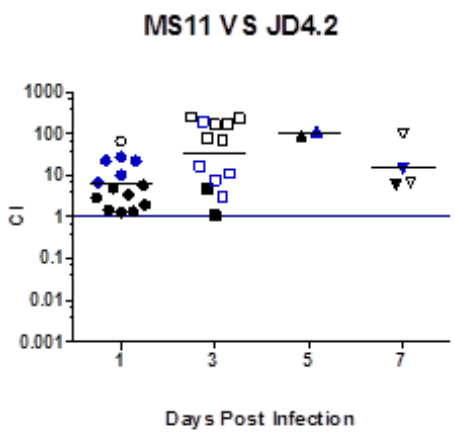
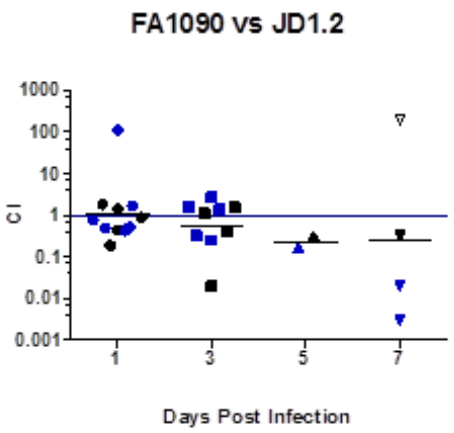

A
1.5
1.0
0.5
0.0
A600
0 1 2 3 4 5 6 7 8
Time (Hrs)
MS11
FA1090
JD4.2
JD1.2
B
1000
100
10
1
0.1
0.01
0.001
CI
0 2 4 6 8
Time (Hrs)
C
1000
100
10
1
0.1
0.01
0.001
CI
2 4 6 8
Time (Hrs)

Figure 16. In vivo assessment of fitness between parental bacteria and the *gyrA*$_{91/95}$ and *parC*$_{86}$ Cip^{R} mutants

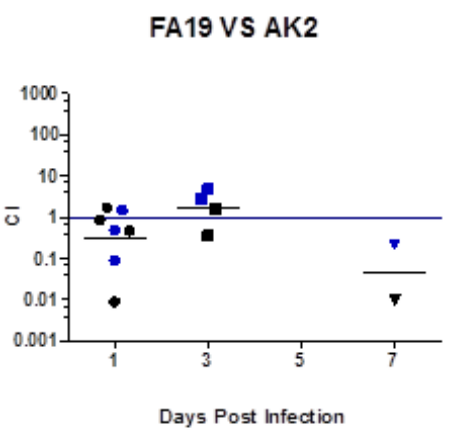
The *gyrA*$_{91/95}$ and *parC*$_{86}$ Cip^{R} mutants in the FA1090 and FA19 backgrounds display an *in vivo* fitness burden in competition experiments with isogenic parents using the mouse model of infection. However, Cip^{R} mutant in an MS11 background exhibits a fitness advantage over parent bacteria. The geometric means of repeat experiments are presented. Black symbols represent the first experiment, and blue symbols represent the second. Open symbols are shown for vaginal cultures yielding only mutant bacteria.



GM CI: 6 - 32



GM CI: 0.2 - 1



GM CI: 0.04 - 1.6

Discussion and Future Directions

The *in vivo* fitness burden of high level resistance to ciprofloxacin in *Neisseria gonorrhoeae* can be overcome through the acquisition of compensatory mutations, and understanding the nature of these mutations will provide a better understanding of specific bacterial mechanisms that can directly contribute to the fitness of *Neisseria gonorrhoeae*. Some of these fitness benefits may act at the level of increased growth and be observed both *in vitro* and *in vivo*, such as was seen for AK13. Others may only occur *in vivo*. The *in vitro* growth of compensatory mutant JD1.4 was not examined and thus at this time, we do not know if the observed fitness restoration occurs only in the context of the host.

Continued investigation of the mutations found in AK13 is needed to understand the basis of the compensatory phenotype. The F91L amino acid and the repaired *mtrR* promoter may be the sole source of the enhanced fitness observed with AK13; however, why repair of the *mtrR* mutation, which is predicted to reduce fitness, contributes to the *in vivo* phenotype of AK13 is not known and it is possible that none of these mutations contributes to enhanced fitness. To address this issue, the GyrA F91L allele could be transferred to the parent strain AK12, followed by repeat *in vitro* and *in vivo* measures of growth. If no evidence is obtained in support of the GyrA F91L being responsible for the compensatory phenotype shown by AK13, whole genome sequencing should be carried out for a more comprehensive analysis. Similarly, growth kinetics and repeat testing of *in vivo* fitness should be conducted to analyze the putative compensatory mutant isolated from the JD1.2, and whole genome sequencing performed if studies confirm the compensatory phenotype. These experiments may reveal new information about

gonococcal adaptation mechanisms as well as identify compensatory mutations that may also be present in Cip^R clinical isolates.

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